BIOLISTIC AND *AGROBACTERIUM*-MEDIATED GENETIC TRANSFORMATION OF IMMATURE AND MATURE EMBRYOS OF SPRING WHEAT CULTIVAR SARATOVSKAYA-29

A Thesis

by

ARMAN A. KOPBAYEV

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2004

Major Subject: Molecular and Environmental Plant Sciences

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Approved as to style and content by:

J. H. Gould (Chair of Committee)	K. Rathore (Member)
Z. J. Chen (Member)	M. Binzel (Chair of Molecular and Environmental Plant Sciences Faculty)
C. T. Smith (Head of Department)	

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ABSTRACT

Biolistic and *Agrobacterium* – Mediated Genetic Transformation of Immature and Mature Embryos of Spring Wheat Cultivar Saratovskaya-29.

(May 2004)

Arman A. Kopbayev, B.S., Zhezkazgan University, Kazakhstan

Chair of Advisory Committee: Dr. J. H. Gould

Plant transformation provides a promising methodology of introducing new genes that encode desirable traits to a wide range of crop plants. Success in genetic transformation has been achieved in many of the important crop species, such as soybean, cotton, rice, corn. However, wheat, one of the major crops of the world, has been considered to be difficult to transform via either *Agrobacterium* or biolistic bombardment (Rakszegi et al., 2001). There have been limited studies on *A. tumefaciens*-mediated transformation of cereals, including wheat, because of the overall refractory character of host-pathogen interactions between *Agrobacterium* and the cereal plants (Gould et al., 1991; Hiei et al., 1994; Cheng et al., 1997). While the genetic transformation of rice using *Agrobacterium* has become routine, only a few successful studies of *Agrobacterium*-mediated transformation of wheat have been reported, and these involved a model spring wheat, *Triticum aestivum* cultivar Bobwhite (Cheng et al., 1997). Model genotypes are developed for ease of plant regeneration in tissue culture and both *Agrobacterium* and biolistic mediated transformation methods require regeneration of plants in tissue

culture. More success has been achieved in obtaining fertile transgenic wheat plants by particle bombardment, or biolistics method (Vasil et al., 1992; Weeks et al., 1993; Becker et al., 1994; Zhou et al., 1995; Altpeter et al., 1996). Wheat plants of the model system cultivar Bobwhite were used in most of these studies as well.

The primary objective of this study was to use the callus-based transformation procedures mentioned above with a non-model cultivar of hexaploid spring wheat Saratovskaya-29, widely grown in Kazakhstan, to test the genotype dependence of the previously developed transformation protocols with respect to stable transfer of DNA and regeneration of transgenic plants. The spring wheat cultivar Saratovskaya-29 (Albidum-24/ Lutescens-55-11) was chosen for the study as being one of the most widely grown wheat cultivars both in Russia and Kazakhstan. It was bred in early 50's in the Research Institute of the South-East, Saratov. Because of its drought resistance and good baking quality traits, Saratovskaya-29 reached a peak of nearly 21.2 mln ha in the former USSR in 1996 (Martynov and Dobrotvorskaya, 1996). Economical importance of this cultivar makes it an appropriate candidate for further improvement of economically significant traits. Another objective of the study described was to compare the transformation efficiencies and inheritance in the transgenic plants produced.

This thesis is dedicated to my family.

To my Dear Father, the most influential person in my life, who showed me what the attitude towards life's responsibilities should be.

To my Beloved Mother, who brought me into this world and made me who I am with constant dedication and unconditional support.

To my Brother, who was many times a teacher for me, being in fact my junior, and provided absolute help and support during my time here and all the times before.

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CHAPTER I

INTRODUCTION

WHEAT AND METHODS OF PLANT TRANSFORMATION

World Wheat Production and Importance

Total world wheat production for 2003/04 was forecast to be 2.292 billion bushels (USDA, August 2003). Although this global wheat production was approximately 32 million tons lower than in the previous year, wheat remains the world's most cultivated grain crop. Historically, increase of world wheat production between 1943 and 1978 averaged 3.3% per year. The production increase at the beginning of that period was due to both expansion of production area and increased per acre yields. In the 1960's yield increase was due to improved varieties and extensive use of irrigation, pesticides and fertilizers. The great impact of these new varieties was called the "green revolution". Between 1982 and1991, the rate of wheat production slowed down to 1.5% per year. The present decreasing tendency in the world's production of wheat could be characterized by the fall in wheat production in the so-called traditional exporting countries, such as US, Australia, Canada, Europe and Argentina. For example, US wheat production decreased 30% in the 3 years to 2003, while wheat consumption in the world has increased from 549,272 tons in 1999/2000 to 565,483 in 2002/2003 and is forecast to increase further (USDA, August 2003).

This thesis follows the format and style of the journal Plant Physiology.

On the other hand, countries such as India, Russia and Ukraine are becoming more significant wheat exporters; however, the grain exported by these countries is of low quality characterized by inadequate flour strength (P value, hard wheat cultivars have higher P values in comparison with extensibility L values) and extensibility (L value, high L values characterize soft wheat cultivars), which make them unsuitable for use in traditional items as French style bread, pastry, cookies and crackers (US wheat associates/Wheat letter 17 January 03). Both problems of decreased wheat production and low cultivar quality produced by developing countries might be partly resolved through the introduction of new genetically modified varieties with desirable traits introduced by means of plant transformation methods.

Genetics of Wheat

Wheat represents the *Poaceae* (alternative name-*Gramineae*) family and the genus *Triticum.* According to different classifications, number of species in the genus varies from 5 to 27 (Merezhko, 1998). In some classifications, this genus includes species of wheat and *Aegilops* (Morris and Sears, 1967). This genus includes diploid (n=14), tetraploid (n=28) and hexaploid (n=42) species. Bread wheat (*Triticum aestivum)* is hexaploid (AABBDD). Other economically significant species are tetraploid *T. durum* and *T. compactum*. Three groups of polyploids are recognized among wheat species (Zohary and Feldman, 1962); species in each group have one genome in common and differ in other genomes. *T. aestivum* (AABBDD) belongs to the group A along with the tetraploids *T. turgidum* (AABB) and *T. timopheevi* (AAGG). *Triticum* polyploids behave

as genomic amphidiploids; their chromosomes pair in a diploid-like fashion and the mode of inheritance is disomic. The diploid-like behavior of *T.aestivum* is due to suppression of pairing of homeologous chromosomes by the *Ph1* locus on the long arm of the chromosome 5B (Riley and Chapman, 1958). *T. aestivum* contains 2 genomes homologous with the A and B genomes of *T. turgidum*. *Aegilops tauschii* is the most likely donor of the D genome (Morris and Sears, 1967). The B genome donor is not identified conclusively. *T. speltoides* (genome S) appears to be the most likely candidate; screen of the *speltoides*-specific sequence against the genomes of tetraploid and hexaploid wheat allowed to suggest existence of related, but modified B genome in hexaploid wheat compared to modern *T.speltoides* (Daud and Gustafson, 1996). It was hypothesized that B genome could have differentiated from the G genome of *T. timopheevi,* or that both B and G genomes are modified S genomes, coming from an initial amphidiploid (AASS), which may have undergone exchange of the chromosome segments with other amphidiploids or diploids, such as *T. longissima* (genome S^1) or *T.bicornis* (genome S^b) (Feldman et al., 1995). Hybridization of *T. monococcum* (var. *boeoticum*) and *T. speltoides* is believed to give origin to the tetraploid wheat group. Hexaploid wheat is believed to have arisen about 5000 years ago, when genomes of tetraploid wheat (*T. turgidum,* 2n=28, AABB*)* and Asian goatgrass (*A.tauschii,* 2n=14, DD) were combined via amphidiploidisation. The tremendous variability of hexaploid wheats suggests that numerous hybridizations involving different genotypes of *A.tauschii* may have taken place. Dvorak et al. (1998) investigated polymorphism of the restriction fragments at 53 single-copy loci, the rRNA locus *Nor3* and high-molecular

glutenin locus *Glu1* in the D genome of *T.aestivum* and *A. tauschii.* It was found that D genomes of investigated forms of *T. aestivum* are closely related to the gene pool of *A.tauschii* ssp. *strangulata,* from Transcaucasia and southwestern Caspian Iran, and all investigated *T.aestivum* forms appear to share a single D genome gene pool, which is contrary to the hypothesis that several *A.tauschii* parents were involved in the evolution of *T.aestivum* (Dvorak et al., 1998).

Many wild perennial species closely related to wheat; such genera as *Aegilops, Agropyron, Eremopyron* and *Haynalidia* might be mentioned. Along with wheat and rye (*Secale*) they form subtribe *Triticinae* of the tribe *Triticeae* of the grass family (Simmonds, 1976). Wild species are important sources of many traits which may aid genetic diversity of wheat. Numerous attempts of wide interspecific hybridization between wheat and the wild relatives have been made; however, this approach has not been successful due to low affinity between homologous chromosomes of crossed species that leads to poor chromosome pairing and sterility of the progeny. Measures such as embryo rescue, ovule culture, protoplast fusion and grafting along with use of bridging species allowed production of fertile hybrids containing new introgressed alleles. Hybrids of wheat and *Aegilops, Agropyron, Thinopirum, Elymus, Leymus* have been obtained; production of hybrids between wheat and more than 50 wild perennial species was confirmed (Sharma, 1995). Many resistances and morphologically beneficial traits have been transferred to wheat by means of wide hybridization: examples include transfer of disease resistance to *Puccinia recondita* from *Aegilops umbellulata* to wheat by backcrossing nulli 5B amphidiploid hybrids of *T. aestivum/A.*

umbellulata to wheat (Riley, Kimber, Law, 1967) and resistance to Cephalosporum stripe disease, obtained by the *T. aestivum/Thinopyrum ponticum* hybrid AT3425 (2n=56) (Manthre et al., 1985). Recent wide hybridization studies have considered transfer of the various resistance genes from wheat to its wild relatives (Zemetra et al., 1998; Gressel, 2000). With the advancement of plant transformation methods, development of systematic approach for preventing possibility of such transfer is important.

Transgenic Technology in the Wheat Research

Several methods developed by traditional breeding, such as direct hybridization of plants with adapted germplasm, marker-assisted introgression or induced mutagenesis have been used in the past for the generation of genetic variability in the wheat. Gene transfer approaches, developed on the basis of latest achievements in molecular biology, provide new opportunities to increase wheat genetic diversity through the transfer of beneficial genes from virtually any organism. Transgenic technology overcomes the limits of traditional breeding both in terms of the spectrum of potential gene donors and the possibility of introducing only the desired novel genes. The introduction of genes to produce genetically modified crops (GM) may lead to improvement of the yield quality and reduction of yield losses through weed, pests, and pathogen factors. Improvement in the quality of yield may be achieved through modification of dough quality, dietetic traits of proteins and increase in the yield of micronutrients, such as zink, iron, and vitamins. Reduction of yield losses may be achieved by introduction of various genes

conferring resistance to diseases, pests, or abiotic stresses. It should be noted that production of transgenic plants is a complex procedure, including introduction of foreign DNA into host cells, integration of foreign nucleotide sequences into the host genomic DNA, expression of new genes in a controlled way, and stable inheritance of the new trait (Rakszegi et al., 2001).Transgenic methodology promises increase in the genetic variability of wheat, in a ways impossible through traditional breeding.

Methods of Gene Transfer to the Cereals

All currently used cereal transformation techniques are divided into direct gene transfer methods (protoplast-based method, particle bombardment), and *Agrobacterium*-mediated gene transfer, standing alone as a method based on the naturally evolved ability of pathogenic *A. tumefaciens* to transform a plant host during infection (Rakszegi et al., 2001). For successful transformation to occur, the transferred gene must be incorporated into a chromosome of the target plant cell and faithfully copied through successive mitoses. It is important that the transformation event(s) also be heritable, that is, incorporated into the plant's germline and inherited by the plant's progeny. To achieve a successful transformation, a set of criteria must be met, including: 1) competence of target tissues for propagation or regeneration; 2) availability of agents for selection of transgenic tissues; 3) ability to recover transgenic plants at a reasonable rate; 4) a simple, efficient, genotype-independent and cost effective transformation process; 5) tight timeframe in culture to avoid somaclonal variation (tissue culture derived mutations, often producing sterile plants) (Hansen and Wright, 2001). Currently, three

methods appear to fulfill these criteria: protoplast-based transformation, biolistics or microprojectile bombardment, and *Agrobacterium*-mediated transformation. Whichever transformation technique is used, a so-called 'transformation model' system, a plant species or cultivar that is amenable to *in vitro* culture is used first, and method is later extended to elite genotypes. For wheat, the cultivar "Bobwhite' represents such a model system, and it was successfully used in transformation experiments.

Protoplast-Based Method of Gene Transfer

Plant-protoplast based gene transfer was the first method to be developed for introducing foreign genetic material into plant germplasm. Initial step of this technique is isolation of protoplasts of plant cells by mechanical or enzymatic removal of the cell wall. The DNA of interest is subsequently added to the protoplast suspension that is then treated to encourage uptake. Some foreign DNA may be taken up by the cells and some is incorporated into the plant's genome. Protoplasts can be transformed by *Agrobacterium* or by direct transfer methods, facilitated by polyethylene glycol treatment, electroporation or liposomes (Shillito, 1999). Protoplasts can be obtained from an established suspension cell line of callus initiated from immature embryos, immature inflorescences, mesocotyls, immature leaf bases and anthers. The major drawback of the method is low regeneration ability of protoplasts along with the extreme genetic specificity. Cereal suspension cultures were shown to lose their embryogenic potential (DiMaio and Shillito, 1989) and accumulate genetic abnormalities (Karp, 1991) when in culture for a long period of time. Among major crops, this method was shown to

be reproducible in rice and maize (Birch, 1997; Golovkin et al., 1993). In several studies sterile transgenic rice plants were obtained; observed abnormal ploidy was suggested as a source of sterility (Chair, Legavre, Guiderdoni, 1996). For wheat, several stably transformed suspension cell cultures were obtained (Vasil et al., 1991), but attempts to regenerate plants from those cultures were unsuccessful.

Genetic transformation by electroporation might be considered as a derivative of protoplast-based transformation method. High-voltage electrical pulses allow uptake of the foreign DNA through cell membranes from a surrounding buffer solution. Recently, optimum conditions for DNA transfer into mature embryos of barley via electroporation were developed (Gurel, Gozukirmisi, 2000). Electroporation was used successfully along with PEG treatment for the protoplast-based transformation of maize (Fromm et al., 1986). However, this method also has disadvantages, particularly critical importance of target tissue preparation and lower amounts of foreign DNA delivered into the target cells in comparison with microprojectile bombardment; similar problem of the integration of multiple copies of the foreign genes may occur (Rakszegi et al., 2001).

Biolistic (Microprojectile Bombardment) Method

Biolistics, or microprojectile bombardment method of plant transformation was introduced back in the late 1980's (Sanford, 1988). This method is based on the delivery of gold or tungsten particles coated with DNA of interest into the target cells by acceleration. The acceleration can be provided by gun powder, by gases, such as helium or CO^2, or by an electric discharge. Any kind of plant tissue could be used as an explant

for microprojectile bombardment, which is an advantage over other methods; however, regeneration of bombarded tissues into fertile plants can be problematic. Therefore, even though the choice of the target tissue is unrestrained, one with the higher regeneration ability *in vitro* is preferable. Another advantage of this method is that there are no major biological barriers such as those present in case of *Agrobacterium* or protoplast-based transformation (Rakszegi et al., 2001). Biolistics is currently the most widely used method for direct gene transfer and by far the most reliable for the production of the fertile transgenic wheat plants (Rakszegi et al., 2001). Numerous research groups have obtained transgenic wheat plants of T^0 generation and confirmed T^1 progeny using microprojectile bombardment (Vasil et al., 1992; Weeks et al., 1993; Nehra et al., 1994; Becker et al., 1994; Altpeter et al., 1996; Zhang et al., 2000; Wright et al., 2001). Several genes of agronomic importance have been incorporated into wheat, such as rice chitinase gene (Chen et al., 1998), a barley-seed class-II chitinase (Bliffeld et al., 1999), the stylbene synthase gene (Leckband and Lorz , 1998) , the barley yellow mosaic virus coat protein gene (Karunaratne et al., 1996), high-molecular weight (HMW) glutenin subunit genes (Altpeter et al., 1996a; Blechl and Anderson, 1996), and a barley trypsin inhibitor gene (Altpeter et al., 1999). Alterations in the standard transformation protocol, such as preculture of the explant material, use of smaller size microprojectile particles and osmotic pretreatment of the target tissue have yielded improvement in transformation efficiencies (Finer et al., 1999). Effect of the different DNA/gold precipitation processes, types and sizes of particles and tissue culture variables on the transformation efficiency was extensively studied (Rasco-Gaunt et al., 1999).

Infertility and transgene silencing might be named as the major drawbacks of the biolistics technique. General use of tissue-culture responsive, but agronomically less desirable 'model' genotypes also limits applicability of the method, along with somaclonal variation mutations that are often induced in tissue culture. Mechanisms of transgene silencing are not clearly understood; it is often observed when multiple copies of a transgene are integrated, or when inserted genes contain sequence homology to an endogenous gene (Muller et al., 1996). Study on the inheritance and stability of an *Act1D-uidA::nptII* expression cassette of the spring wheat cultivar 'Fielder' has shown relation between high methylation and loss of transgene activity (Demeke et al., 1999). Authors speculated that multiple integrations of the transgene may have triggered transgene methylation followed by transgene silencing and distortion of segregation ratios. Anti-sense RNA production and heterochromatization of the transgenic locus have also been suggested as mechanisms possibly involved in transgene silencing. Chen et al., (1998) observed loss of the rice chitinase *chi 11* gene expression in T^1 progeny of wheat transgenic plants, while *bar* gene expression was unaffected. Since *chi11* gene was driven by CaMV35S promoter and *bar* gene by ubiquitin promoter, authors suggested that selective inactivation of the CaMV35 S promoter may have taken place. Interestingly, the same promoter was effective in T^1 progeny of transformed rice (Hiei et al., 1994), indicating that the CaMV35S promoter is more prone to silencing in wheat, than in rice. Transgene silencing may be minimized by careful choice of the promoter and reporter gene constructs, use of matrix-associated regions (MARs) or scaffold attachment regions (SARs) to insulate transgenes from surrounding chromatin, and use

of *Agrobacterium*-mediated transformation method, which tends to result in low copy transformation and simple integration patterns (Demeke et al., 1999).

Agrobacterium-Mediated Gene Transformation Method

Agrobacterium tumefaciens is a soil bacterium which belongs to the genus *Agrobacterium* of the family *Rhizobiaceae,* which includes both saprophytic and pathogenic bacterial species. Interestingly, recent findings of the 16sDNA sequence similarities suggest that *Agrobacterium* and *Rhizobium* to be combined into one monophyletic clade (Young et al., 2001), although this suggestion is disputed (Farrand et al., 2003). *Agrobacterium tumefaciens* is a pathogenic bacterium that has a naturally evolved mechanism to transfer genes into the chromosomes of host plant cells. To date *Agrobacterium* appears to be the only known organism capable of performing permanent gene transfer to members of other taxonomic kingdoms. Crown gall is a disease naturally occurring among perennial plants caused by *Agrobacterium*. Specific segment of the Ti-plasmid, T-DNA, might be engineered by initial disarming (removal of the bacterial tumorigenic genes contained in the T-DNA) and insertion of a selectable marker and genes of interest. Other important factors required for successful *Agrobacterium*-mediated transformation are the bacterial virulence (*vir)* genes, regulating mechanism of host inoculation, and phenolic inducers of virulence, such as acetosyringone, that are released by wounded plant cells, triggering expression of the *vir* genes and initiating transformation process. *Agrobacterium*-mediated transformation is advantageous in terms of the relative simlplicity of the protocol and minimal equipment

cost. The yields of transgenics obtained are usually 10-30%, the transferred genes are often present in single or low copy, incorporated in a stable manner, and inherited in the plant's progeny (Gould, 1997). It was thought that *Agrobacterium* was incapable of transferring large (>10 kb) tracts of DNA, until it was demonstrated that the BAC fragments of 120-150 kb could be transferred (Hamilton et al., 1996).

Until recently it was believed that *Agrobacterium*-mediated plant transformation could only be used successfully with dicotyledonous plants, being natural hosts of *A.tumefaciens*; monocotyledonous plants including cereals were excluded from the range of *A.tumefaciens* hosts partly due to demonstrated inability to produce signaling compounds for activation of *vir* genes (Usami et al., 1987) and lack of a typical wound response. But the idea of principal possibility of DNA transfer to monocot plants via *A.tumefaciens* has found its enthusiastic investigators. Maize was the first of the cereal species shown to be susceptible to *Agrobacterium* infection (Graves and Goldman, 1986; Grimsley et al., 1987). Later, it was shown that use of dividing meristematic cells as target cells for transformation (Gould et al., 1991) along with addition of *vir* inducing compounds, such as acetosyringone and nopaline in maize (Gould et al., 1991) and potato wound exudate for yam (Schafer et al., 1987) and rice (Chan et al., 1993) leads to successful T-DNA transformation and expression in monocotyledons along with inheritance of transferred genes. Choice of meristematic cells as target cells might be explained by their critical importance (Gould et al., 1991; Chan et al., 1993; Delbreil et al., 1993). Another advantage of meristematic cells is general ability of other types of monocotyledonous cells to lose dedifferentiation ability at early developmental stages,

which might lead to decrease in response to bacterial infections (Graves et al., 1992); that might serve as an explanation for past unsuccessful attempts to transform monocotyledons via stem and leaf tissue inoculations. Finally, meristematic cells might secrete compounds that induce the *vir* genes. Particular advantage of shoot apical meristem-based *Agrobacterium* transformation was that of genotype-independent plant regeneration and avoidance of passage through callus intermediate and somaclonal variation mutations, which impact fertility, yield and qualitative traits (Gould, 1997).

The 1990's and current decade might be considered as a breakthrough period in the transformation of cereal plants. Success in *Agrobacterium*-mediated transformation of maize (Gould et al., 1991; Ishida et al., 1996; Zhao et al., 2002), rice (Rainieri et al., 1990; Chan et al., 1993; Hiei et al., 1994; Aldemita and Hodges, 1996; Kant et al., 2001; Ming et al., 2001), and barley (Tingay et al., 1997) was achieved. Hiei et al. (1994), used superbinary vectors pLG121Hm and pTOK233, having additional copies of *vir* genes outside the T-DNA region on the binary vector, and immature embryos as explant source. The authors proved stable transformation of the rice plants of cv *japonica* by use of the sequence analysis of the plant/T-DNA border junction regions. Cheng et al. (1997), Khanna and Daggard (2003), Hu et al. (2003) achieved successful transformation of wheat with obtainment of T^0 transgenic plants and T^1 transgenic progeny. Work of Khanna and Daggard is of particular interest; using superbinary vector pHK21 with an extra set of *vir* genes outside the T-DNA region and polyamine spermidine as a principal supplement of regeneration media, they were able to obtain 17 stably transformed plants of spring wheat cv Veery 5 from 587 immature embryo-

derived calli, when the same procedure with ordinary binary vector recovered no transformants. Authors suggest that use of superbinary vectors containing additional copies of inductive *vir* genes might be a critical factor in the development of high-efficiency transformation protocols.

Various factors influence bacterial and plant cells involved in a process of *Agrobacterium* infection. These factors need to be analyzed and optimized for each species. Wu et al. (2003) have analyzed set of factors influencing *Agrobacterium*-mediated transformation of wheat, and discovered that such factors as embryo size, duration of preculture, inoculation and cocultivation, and the presence of acetosyringone and Silwet L-77 in the media induced significant differences in T-DNA delivery and regeneration. Authors demonstrated that conditions favoring T-DNA delivery are not necessarily the same as those favoring the recovery of the stable transformation events. For example, T-DNA delivery was shown to increase with shorter pre-culture times, longer inoculation and higher Silwet L-77 concentrations, whereas the ability of the explants (immature embryos) to survive cocultivation increased with exactly the opposite condition changes applied. Applications of 0.01% Silwet L-77 and 200μ*M* acetosyringone were shown to increase T-DNA delivery without losing the regeneration potential of the immature embryos in the four wheat varieties tested (spring wheat: *Bobwhite, Canon;* winter wheat: *Florida, Cadenza*). Methodology of *Agrobacterium*-mediated wheat transformation is still under development; immature embryo-derived calli appear to be the preferred tissue source. Attempts to use other types of explants for *Agrobacterium*-mediated transformation, as inflorescence tissue, weren't successful

(Amoah et al., 2001). The most efficient application of *Agrobacterium*-mediated transformation was achieved with limited range of model genotypes, of which BobWhite shows higher amenability to *Agrobacterium* infection. Use of superbinary vectors for inoculation, careful formulation of regeneration media to overcome decrease in regeneration response from inoculated tissue culture explants, and optimization of factors influencing *Agrobacterium*-mediated transformation, such as timing of inoculation and cocultivation, and concentration of acetosyringone and surfactant applied, appear to be main means of increasing effectiveness of *Agrobacterium*-mediated transformation of wheat. Of interest are attempts to combine *Agrobacterium*-mediated and particle bombardment procedures; for example, bombardment of apical meristemes of tobacco cv. Xanthi and sunflower was used for wounding before applying *Agrobacterium* solution, and this procedure was more effective in inducing transient GUS expression then standard gene gun bombardment protocol (Bidney et al.,1992). Similar approach was successfully used for the *Agrobacterium*-mediated transformation of recalcitrant rice cultivar Zhonghua 8 (ssp. *japonica*) (Ming et al., 2001).

CHAPTER II

BIOLISTICS AND *AGROBACTERIUM*-MEDIATED GENETIC TRANSFORMATION OF IMMATURE AND MATURE EMBRYOS OF SPRING WHEAT CULTIVAR SARATOVSKAYA-29

INTRODUCTION

Agrobacterium-Mediated Wheat Transformation

As it was noted earlier, monocotyledonous plants were considered recalcitrant for *Agrobacterium*-mediated transformation. However, this notion was questioned when reports indicating *Agrobacterium*-mediated gene transfers in non-cereal monocot plants asparagus (Hernalsteens et al., 1984) and dioscorea (Schafer et al., 1987) appeared. Further reports demonstrated that the cereals, maize (Graves and Goldman, 1986; Gould and Smith, 1989; Gould et al., 1991) and rice (Raineri et al., 1990; Chan et al., 1993) could also be transformed via *Agrobacterium.* Gould et al. (1991) reported the first production of transgenic cereal plants (maize) and the production of transgenic progeny, demonstrating at the molecular level that *Agrobacterium*-mediated transformations of a cereal were stable and inherited. Chan et al. (1993) first reported successful production of transgenic rice plants by inoculating immature embryos, and performed molecular and genetic analysis of the progeny to prove the stable, heritable transformation. Further demonstration of successful transformation of cereals mediated by *Agrobacterium* was marked in the papers on the rice transformation (Hiei et al., 1994) and maize (Ishida et al., 1996). These papers provided the data that allowed accepting the concept and helped

to establish routine *Agrobacterium*-mediated transformation protocols. However, studies on *Agrobacterium*-mediated transformation of wheat (*Triticum aestivum* L.) were limited and less reproducible. Hess et al. (1990) attempted pipetting of *Agrobacterium* solution into spikelets of wheat, but the protocol used was not readily reproducible, and inheritance of the new construct was not proved. Deng et al. (1990) were among the first to demonstrate formation of opine-synthesizing tumors on infected bases of leaves and spike stems of wheat. But achieving efficient stable transformation was problematic and demanded a set of criteria to be met.

According to a popular review (Potrykus, 1991), criteria on which success of transformation process is strongly dependent are genotype, explant source, and medium composition. The choice of the spring wheat cultivar Saratovskaya-29 for this study was based on its wide distribution and economical importance both in Kazakhstan and Russia. In addition, Saratovskaya-29 was one of the most common parents of spring wheat cultivars released in the former U.S.S.R. between 1980 and 1996, with ancestral contribution for Kazakhstani cultivars reaching 26.5% (Martynov and Dobrotvorskaya, 1996). Progeny spring cultivars of Saratovskaya-29 would be expected to respond similarly to transformation and tissue culture procedures developed for this cultivar. Choice of the immature embryo-derived calli as an explant source for this study was based on previous successes in *Agrobacterium*-mediated transformation using this type of explant (Cheng et al., 1997; Khanna and Daggard, 2003). Mature embryo-derived calli, that developed directly from the precultured mature seed, were also used in this study. Callus-based methods for plant regeneration were considered appropriate for the

use in the study, because of the responsiveness of the Saratovskaya-29 cultivar during the development *in vitro* for callus growth and organogenesis from unfertilized ovaries (Kopbayev et al., 2000). *A. tumefaciens* strain carrying binary vector pTOK233 (Hiei et al., 1994) was used in *Agrobacterium*-mediated transformation, and the pAHC25 plasmid (Christensen, Quail, 1996) was used in and gene gun bombardment transformation procedure. Both constructs were used successfully for the transformation of the cereal crop plants (Hiei et al., 1994; Ratnayaka, 1999, Atpeter et al., 1996). The plant growth regulator 2, 4-dichlorophenoxyacetic acid (2, 4-D) was chosen for the callus induction medium and zeatin for the regeneration medium. These growth regulators were used previously for the transformation of wheat (Altpeter et al., 1996; Cheng et al., 1997), and 2, 4-D was already shown to induce callus growth for Saratovskaya-29 (Kopbayev et al., 2000).

The objectives of the study were: 1) transform and regenerate plants of elite non-model spring wheat cultivar Saratovskaya-29 using *Agrobacterium*-mediated and biolistic methods of transformation; study 2) transient expression of transferred marker gene (*UidA* in both cases); 3) transformation efficiency and 4) inheritance of the foreign genes.

MATERIALS AND METHODS

Preparation of Plant Material and Culture

Seeds of the spring wheat cultivar Saratovkaya-29 were obtained courtesy of Dr. Mukhambetzhanov (Almaty State University, Kazakhstan). Procedures used for both

Agrobacterium and biolistic gene transfer operations are summarized in the Figure on p. 34. Calli initiated from mature seeds and immature embryos were used in this study as an explant source. Particle bombardment transformation was performed on the same group of calli that were used for *Agrobacterium*-mediated transformation (Dai et al., 2001).

Plant Growth Conditions, Isolation and Callus Induction Culture

Wheat plants (*Triticum aestivum* L., cv. Saratovskaya-29) were grown in a growth chamber at 20˚C/15˚C day/night temperature and 16 h photoperiod at 1000 μE-m^2-s^{-1}. Immature embryos were collected from flower spikes 10 to14 days after anthesis, and mature embryos were collected in 7 to 10 days after the grain filling. Spikes were used fresh both for the isolation of immature or mature embryos. For isolation of immature embryos, immature seeds were surface sterilized with 70% ethanol for two min. and 25% Chlorox with 0.1% Tween 20 for 25 min, followed by a rinse of four changes of sterile distilled water. For isolation of mature embryos, seeds were surface sterilized with 70% ethanol for two min., subjected to 70% Chlorox with 0.1% Tween 20 for 1 h, then to 30% hydrogen peroxide for 1h, followed by four rinses of sterile distilled water. Immature embryos (0.5-1.5 mm in size) were aseptically removed with forceps under sterile conditions in the laminar air- flow hood and placed scutellum side up on MS medium (Murashige and Skoog, 1962) supplemented with 2 μg/mL^{-1} 2,4-D, 20 μg/mL^{-1} sucrose, 500 μg/mL^{-1} glutamine and 100 μg/mL^{-1} casein hydrolysate (MS+ medium) (Altpeter et al., 1996). The same isolation procedure was applied to mature embryos.

Callus induction medium, described above, was used both for *Agrobacterium* and particle bombardment procedures. Both immature and mature embryos were precultured in the dark on the callus induction medium for 3 to 5 days prior to the transformation.

Agrobacterium Strain and Culture Condition

The *Agrobacterium* strain LBA 4404 (TOK233) was grown using two pre-inoculation procedures developed for *Agrobacterium*, by Ratnayaka (1999) and by Lichtenstein and Draper (Lichtenstein and Draper, 1985). In the first of pre-inoculation procedures mentioned (Ratnayaka, 1999), *Agrobacterium* strain LBA 4404 (pTOK233) was grown 10-24 hours on LB media (Sigma, St. Louis) solidified with 15 g of Bacto Agar at pH 7.0 (GIBCO BRL, Gaithersburg, MD), containing hygromycin (50 μg mL^{-1}) (Sigma, St. Louis, MO). Bacteria were then removed from the plate using the glass spatula and suspended in *Agrobacterium* activator medium (AAM), (Hiei et al., 1994), virulence activator acetosyringone was added at 100 μM immediately before inoculation. The optical density (OD) of the bacterial suspension was measured using a spectrophotometer and OD value of 0.6 was taken as the optimal concentration for inoculation. In the second procedure, *Agrobacterium* strain LBA 4404 (pTOK233) was grown overnight on the solidified LB medium containing 50 μg mL^{-1} hygromycin. A single bacterial colony was removed using a toothpick and suspended in a liquid LB medium containing 50 μg mL^{-1} hygromycin, at volume of 2 ml per tube. Culture tubes were incubated at room temperature, and placed on an orbital shaker for 12-16 hours at 1600 rpm. The resulting bacterial suspension was added into 10 ml of pre-induction

medium (PIM), acetosyringone added at 2 µl/mL^{-1}, and incubated on a shaker for an additional 10-12 hours (Lichtenstein and Draper, 1985) before inoculation.

Plasmids

The hybrid binary vector pTOK233 is the cointegrated form of the two plasmids pTOK162 and pGL2-IG generated by homologous recombination (Komari, 1990 b). T-DNA region of binary vector pTOK233 is shown in Fig.2-1 and vector pAHC25 is shown in figure 2-2.

The T-DNA region of pTOK 233 plasmid contains genes *Npt, Hpt* and *UidA* genes (Hiei et al., 1994). The beta-glucuronidase gene (*UidA)* encoding beta-glucuronidase GUS enzyme (Jefferson, 1987) was modified to contain an eukariotic intron in the N-terminal region of the coding sequence; this intron was fused with the 35S promoter of cauliflower mosaic virus (Vacancy et al., 1990). The role of this intron is to enhance expression of the *UidA* gene by eukaryotic plant cells and inhibit expression of *UidA* by *Agrobacterium* by occupying the prokaryotic binding site in the promoter. The *Agrobacterium* strain LBA4404(pTOK233) was supplied courtesy of Japan Tobacco Inc., Japan.

The plasmid pAHC25 (Christensen and Quail, 1996) was used in particle bombardment transformation procedure, courtesy of Dr. PH Quail (USDA, Albany CA). This plasmid contains the selectable *bar* gene, encoding the enzyme phosphinothricin acetyltransferase (PAT) and the GUS gene (*UidA)* encoding ß-glucuronidase, both

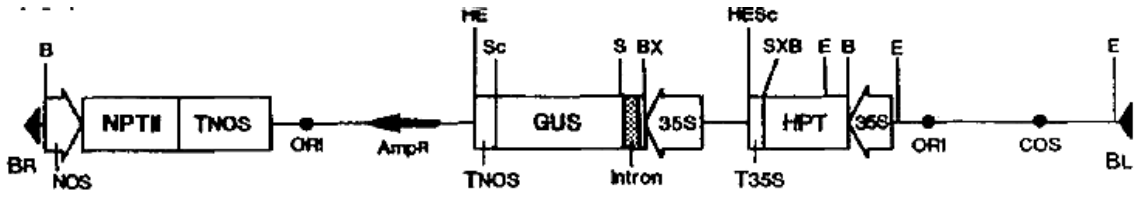

Figure 2-1. T-DNA region of plasmid pTOK233 (redrawn from Hiei et al., 1994). Abbreviations: BR, right border; BL, left border; *NptII,* neomycin phosphotransferase II; *GUS,* ß-glucuronidase (*UidA)*; *Hpt,* hygromycin phosphotransferase; NOS, nopaline synthase promoter; 35S, Cauliflower Mosaic Virus promoter (CaMV35S); TNOS, 3' eukaryotic terminator signal of nopaline synthase (NOS); T35S, 3' eukaryotic terminator signal of 35S RNA.

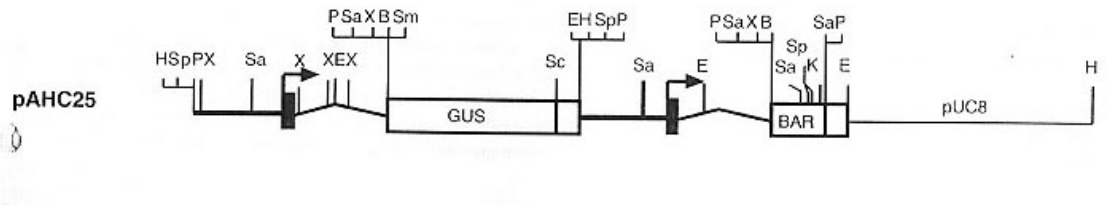

Figure 2-2. Schematic diagram of the vector pAHC25 (redrawn from Christensen and Quail, 1996). Bold straight line, *Ubi-1* promoter sequences; filled box, *Ubi-1* exon; angled line, *Ubi-1* intron; blank open box, nopaline synthase 3'untranslated sequence; thin straight line, pUC8 sequence. Arrow at the *Ubi-1* exon signifies transcription start site. *GUS,* ß-glucuronidase; *BAR,* phosphinothricin acetyltransferase.

driven by separate maize ubiquitin (*Ubi-1)* promoter (Christensen and Quail, 1996). The plasmid was constructed by excising the Ubi-GUS-NOS containing *Hin*dIII fragment from pAHC27 and subcloning it into *Hin*dIII-digested UBI-BAR containing pAHC-20 plasmid (Christensen and Quail, 1996).

Agrobacterium Co-cultivation Procedure

Calli developed from both immature and mature embryos were collected into 50 ml sterile test tubes (Cell Star, Greiner Bio-one), and the *Agrobacterium* suspension in AAM medium (Hiei et al., 1994) or in PIM medium (Lichtenstein and Draper, 1985) was added. The tubes were placed under vacuum for 5 min (ROC-R Vacuum-MFG Corp., Benton Harbor, MI). Calli were then plated on filter paper on co-cultivation medium (half-strength MS salts plus 3% sucrose) in darkness for 2-3 days at 22°C.

Particle Bombardment Procedure

Preparation and delivery of DNA-coated gold particles was performed according to Vasil and Vasil (Vasil V and Vasil IK, 1999). The Biolistic™ Particle Delivery System PDS-100 (E. I. Dupont deNemours & Co. Biotech. System Division, Wilmington, DE) was used in the study. Standard 1100 psi microcarrier disks were used for all experiments. Calli were precultured on high osmotic medium (MS+ medium supplemented with 0.2 M mannitol and 0.2 M sorbitol) for 4 hours prior and 18 hours after bombardment (Altpeter et al., 1996).

Selection and Plant Regeneration

Hygromycin was used for selection of both immature and mature embryo-developed calli in *Agrobacterium*-mediated transformation procedure because the CaMV35S promoter used with the *Hpt* gene is known to be active in callus tissue (Hiei et al., 1994), while the Nos promoter of *NptII* gene is not as active in callus. After co-cultivation, calli were collected into sterile test tubes and washed in an antibiotic solution to kill *Agrobacterium* (Clavamox, Smith Kline-Beecham; Gould, Magallanes, 1999) at concentration of 250 μg mL^{-1} for 15 minutes. Calli were then transferred to MS media supplemented with 2 μg mL^{-1} 2, 4-D and 250 μg/mL^{-1} Clavamox (amoxicillin trihydrate/ clavulanate potassium, Veterinary Research Triangle Park, NC) and cultured for 1 week. After 1 week, calli were transferred to selective callus induction media supplemented with 2 μg/mL^{-1} 2,4 –D, 250 μg/mL^{-1} Clavamox and 50 μg/mL^{-1} hygromycin. After 3-4 weeks, viable embryogenic calli were selected and transferred to the same media for another 3-4 weeks. Calli that survived this treatment, were transferred to regeneration media (MS media supplemented with 0.5 μg/mL^{-1} zeatin and 250 μg/mL^{-1} Clavamox), which did not contain the selective antibiotic, hygromycin. Culture plates were kept at a growth chamber with a 16 h light and 8 h dark photoperiod at 26° C. Developing shoots were transferred to MS media with half-strength salts and vitamins, supplemented with 15 μg/mL^{-1} sucrose and 50 μg/mL^{-1} hygromycin but without any hormones (MS/2) for 1-2 cycles of 14 days each (Altpeter et al., 1996). When rooted shoots were about 15-20 mm long, they were transferred to ‘Jiffy’ peat

pellets and cultured under the 'Ziploc' bags for 5-7 days, then transferred to soil and grown to maturity in a growth chamber under the conditions described above.

In the particle bombardment transformation procedure, selection and regeneration protocol of Altpeter et al. (1996) was followed. After post-bombardment osmotic treatment calli were transferred to callus induction media MS+ (same as in *Agrobacterium* procedure) without the selective agent (bialaphos) (Product No.: B 131, PhytoTechnology Laboratories, Shawnee Mission, KS) for 7 to 10 days. Calli were then transferred to selective callus induction medium (MS+ supplemented with 2 $\mu g/mL^{-1}$ 2,4-D and 4$\mu g/mL^{-1}$ bialaphos) for two subsequent culture cycles ~ 1 month each. Surviving calli have been transferred to regeneration media described above for *Agrobacterium* procedure, without selection. Developing shoots were transferred to MS/2 media supplemented with 5 $\mu g/mL^{-1}$ bialaphos. Once shoots were approximately 15-20 mm long, they were transferred into 'Jiffi' peat pellets for 5-7 days and then transferred to soil. As *Agrobacterium* transformed plants, they were grown to maturity in growth chamber.

Analysis

GUS Histochemical Assay

The GUS (beta-glucuronidase) histochemical assay procedure developed by Jefferson (Jefferson, 1987) was used to observe the expression of the transferred *UidA* (GUS, beta-glucuronidase) gene. Two to three days following bombardment 3 to 4 calli from each plate were collected and treated with X-Gluc (5 Bromo-4-Chloro-3-Indoxyl-beta-D-

glucuronic acid, Cat. 28056/2, Biosynth Ag) in 50 mM Sodium Phosphate buffer (Jefferson, 1987). After adding calli to solution, samples were incubated at 37° C overnight. Tissues were then examined under the microscope, and cells exhibiting active expression of the *UidA* gene were observed as blue colored areas. The GUS histochemical assay was also applied to leaf pieces of the regenerated wheat plants 1 to 2 weeks after the transfer to soil. Leaf samples were surface sterilized (25% commercial bleach for 15 min.) and then treated with X-Gluc with subsequent 37°C incubation overnight. Leaf samples were then placed in 95% ethyl alcohol for 3-4 h and examined under microscope for *UidA* expression.

DNA Isolation

Leaf tissues from young seedlings were extracted for DNA using a urea-phenol extraction method (Shure et al., 1983) and Potassium ethyl xanthogenate (PEX) extraction method (Williams and Ronald, 1994). DNA concentration was measured using a fluorimeter (model TKO 100, Hoefer Scientific Instruments, San Francisco); calf thymus DNA (Sigma, St. Louis) was used as a standard for the estimation.

PCR

UidA Gene

PCR to amplify *UidA* sequences was performed in a volume of 25 µl using a PCR kit (PCR Master kit, Roche Diagnostics Corporation, Indianapolis, IN). Amount of plant DNA samples was adjusted to ~ 100ng/25 μL^{-1} volume reaction. Amplifications were

carried for 30 cycles (denaturation: 94°C, 30 sec.; annealing: 48°C, 1 min.; extension: 72°C, 3 min.). Sequences of the *UidA* primers used for PCR amplification were: primer1: 5'TTCGGTGATGATAATCGGCTGTTCGGTG 3' and primer 2: 5'GGTTATCAGCG CGGAAGTC 3'. Expected fragment size was ~ 1.6 kb for both pTOK233 and pAHC25 plasmids, since primers were taken from the exon sequence of the *UidA* gene in both plasmids. PCR products were analyzed by gel electrophoresis in a 0.6% agarose (Sigma, St.Louis, MO). The size of the amplified fragments were determined with reference to molecular weight marker Lambda DNA/EcoRI+HindIII Markers (Cat.No G173A, Promega, Madison, WI) used as a size standard for fragments larger than 1 kb. Gel was stained with Ethidium Bromide (GIBCO BRL, Gaithersburg, MD) to view fragments.

NptII Gene

PCR amplification of the *NptII* gene was performed in a volume of 25 µl using PCR kit (PCR Master kit, Roche Diagnostics Corporation, Indianapolis, IN). Amount of plant DNA samples was adjusted to ~100 ng/25 μL^{-1} volume reaction. Amplifications were carried for 30 cycles (denaturation: 94°C, 30 sec.; annealing: 48°C, 1 min.; extension: 72°C, 3 min.) Sequences of the *NptII* primers used for PCR amplifications were: primer 1: 5' AGACAATCGGCTGCTCTGAT 3' and primer 2: 5' AGCCAACGCTATGTCC TGAT 3'. Expected fragment size was ~ 593 bp. PCR products were analyzed by electrophoresis in a 0.6% agarose. Size of the fragment was determined with reference to PCR molecular weight markers (Cat.No G316A, Promega, Madison, WI) used as a 1 kb size standard. Gel was stained with Ethidium Bromide (Gibco BRL, Gaithersburg, MD).

Hpt Gene

PCR amplification of the *Hpt* gene was performed in a volume of 25 µl using PCR kit (PCR Master kit, Roche Diagnostics Corporation, Indianapolis, IN). Amount of plant DNA samples was adjusted to ~ 100 ng/25 μL^{-1}volume reaction. Amplifications were carried for 38 cycles (denaturation: 94°C, 30 sec.; annealing: 55°C, 30 sec.; extension: 72°C, 1 min.). Sequences of the *Hpt* primers for PCR amplifications were: primer 1: 5'GAAAAAGCCTGAACTCACCG 3' and primer 2: 5' ACATTGTTGGAGCCGAAA TC 3'. Expected fragment size was ~ 570 bp. PCR products were analyzed by electrophoresis in a 0.6% agarose gel. Size of the fragment was determined with reference to PCR molecular weight markers (Cat. No G316A, Promega, Madison, WI) used as a 1 kb size standard. Gel was stained with Ethidium Bromide (Gibco BRL, Gaithersburg, MD).

Bar Gene

PCR was performed in a volume of 25 µl using PCR kit (PCR Master kit, Roche Diagnostics Corporation, Indianapolis, IN). Amount of plant DNA samples was adjusted to ~ 100 ng/25 μL^{-1} volume reaction. Amplifications were carried for 40 cycles (denaturation: 95°C, 30 sec.; annealing: 52°C, 30 sec.; extension: 72°C, 1 min.). Sequences of the *bar* primers used for PCR amplifications were: primer 1: 5'AGGACAGAGCCACAAACACC 3' and primer 2: 5' AAATCCCTTTGCCAAAAC C 3'. Expected fragment size was ~ 390 bp. PCR products were analyzed by electrophoresis in a 0.6% agarose gel. Size of the fragment was determined with reference to PCR molecular weight markers (Cat.No G316A, Promega, Madison, WI)

used as a 1 kb size standard. Gel was stained with Ethidium Bromide (Gibco BRL, Gaithersburg, MD).

PCR Blotting

DNA samples from putative transgenic plants were PCR amplified using programs described above and run out on agarose gel. After visualization of bands, gel was blotted using alkaline transfer method (Sambrook et al., 1989). DNA was transferred to Hybond-N membrane (Amersham, Arlington Heights, IL) and Southern hybridization (Southern, 1976) was carried essentially according to Sambrook et al. (1989). Pre-hybridization was carried out according to the manufacturer's protocol (Amersham Life Science). Pre-hybridization solution of 10 ml (2.5 ml 20×SSC, 0.5 ml Denhardt solution, 0.5 ml 10% (w/v) SDS, purified H2O added up to 10 ml) was prepared fresh for each procedure. Herring sperm DNA (Cat.No D-6898, Lot 051K7071, Sigma, St.Louis, MO) was denatured for 5min. at 95°C and added to the prehybridization solution at a final concentration of 100μg/mL^{-1}. The blotted membrane-bound DNA was hybridized with random primed ^{32}P probe using Random Prime Labeling Kit (Random Primers DNA Labeling System, Invitrogen Life Technologies, Carlsbad, CA). The same solution was used for hybridization. Prehybridization was performed at 65°C for 4 hours and hybridization was performed at 65°C for 16-18 hours. Membranes were washed in rinsing solutions as follows: 50ml of 2×SSC, 0.1% (w/v) SDS at 65ºC for 25 min., and 0.1×SSC, 0.1% (w/v) SDS at 65ºC for 25 min. Then membranes were placed under X-ray sheets (Kodak X-Omat-AR film, Lot72200902, Eastman Kodak Company,

Rochester, NY), covered with protective sheets and placed in a -70ºC refrigerator. Film was developed 3 to 7 days later, depending on the strength of the hybridization signal.

RESULTS

Hygromycin B Selection of Callus

Calli initiated from mature seeds were plated on MS media supplemented with 2,4-D (2µg/mL^{-1}) and different levels of selection agent hygromycin B (5, 10, 25, 30 and 50 µg/mL^{-}1) after seeds were cultured on MS basic medium (5 calli were placed in each Petri plate). Calli began to show necrotic regions after 3 days in media containing 25, 30 and 50 µg/mL^{-1} hygromycin, and all calli were dead in 9 days on 50 µg/mL^{-1}g hygromycin. Hygromycin B at a concentration of 50µg/mL^{-1} was chosen as an optimum for further selection in *Agrobacterium* mediated transformation experiments.

The herbicide Bialaphos used in the selection of gene gun bombarded plants was applied in concentration of 4µg/mL^{-1}and 5 µg/mL^{-1}, similar to those described by Altpeter et al. (1996).

Regeneration of Plants

Five levels of the cytokinins benzyladenine (BA) and zeatin were tested for effectiveness in shoot initiation, and the concentrations of: 0 µg/mL^{-1}, 0.5µg/mL^{-1}, 3 µg/mL^{-1}, 5 µg/mL^{-1} and 10 µg/mL^{-1} were tested. Immature embryos were precultured on MS medium containing 2, 4-D (2 µg/mL^{-1}) for 15-17 days to obtain viable morphogenic

callus, and then transferred to MS media with given concentrations of cytokinin. Results of the experiment are shown in Table 2-1.

Test showed that MS medium supplemented with 0.5 μg/mL^{-1} zeatin was optimal for shoot initiation. In addition, MS medium containing 0.5 μg/mL^{-1} zeatin was chosen as a medium for shoot initiation in all transformation experiments. MS with half strength salts and vitamins without any selection (MS/2) was used for shoot elongation for about a week, followed by MS/2 medium containing 5 μg/mL^{-1}bialaphos in biolistic bombardment procedure, and by MS/2 containing 50 μg/mL^{-1} hygromycin in *Agrobacterium* mediated transformation procedure. Data on total recovery of plants from immature and mature embryos is summarized in Table 2-2. Seven plants in total were recovered in particle bombardment procedure, four of these originated from immature embryos, and three-from mature embryos (Table 2-2). In *Agrobacterium*-mediated transformation procedure, recovery was achieved with 17 plants, 6 of which originated from immature embryos and 11-from mature embryos (Table 2-2). Protocol for the obtainment of plants of cv Saratovskaya-29 following *Agrobacterium*-mediated and particle bombardment procedures is shown in Figure 2-3.

Table 2-1. *Effect of exogenous benzyladenine and zeatin on regeneration frequency of immature embryos of wheat cv Saratovskaya-29*

Concentration of cytokinin,µg/mL^{-1}	**No of calli**	**No of shoots regenerated**	**Regeneration,%**
0.0	20	4	20.00
Benzyladenine			
0.5	20	4	20.00
3	18	6	33.33
5	20	7	35.00
10	20	2	10.00
Zeatin			
0.5	20	14	70.00
3	20	3	15.00
5	20	4	20.00
10	20	-	-

Table 2-2. *Total number of plants recovered from Agrobacterium and biolistic transformation procedures*

Procedure	Total No. of embryos	No. of plants recovered	Rate: plant recovery
Agrobacterium-mediated transformation			
Mature embryos	452	11	2.43 %
Immature embryos	622	6	0.96 %
Total *Agrobacterium*	1074	17	1.58 %
Particle Bombardment			
Mature embryos	460	3	0.65 %
Immature embryos	526	4	0.76 %
Total particle bombardment	986	7	0.70 %

Figure 2-3. *Culture schedule for wheat cultivar Saratovskaya-29.*

Agrobacterium-mediated procedure			**Microprojectile bombardment procedure**		
Procedure	**Time**	**Medium**	**Procedure**	**Time**	**Medium**
Callus induction	3 to 5 days in the dark	MS+2µg/mL^{-1} 2,4-D, 500µg/mL^{-1} glutamine, 100 µg/mL^{-1}casein hydrolysate (MS+ medium)	Callus induction	3 to 5 days in the dark	MS+2µg/mL^{-1} 2,4-D, 500µg/mL^{-1} glutamine, 100 µg/mL^{-1}casein hydrolysate (MS+ medium)
Inoculation	2-3 days in the dark	Pick a single colony from bacterial plate. Suspend in 2 ml of liquid LB+50µg/mL hygromycin. Incubate on a shaker for 16 h. Add resulting bacterial suspension into PIM medium+acetosyringone at 2µl/mL^{-1}. Incubate on a shaker for 10-12h. Add acetosyringone at 2µl/mL^{-1}before inoculation. MS with half strength salts and vitamins (MS/2)+3% sucrose	Bombardment	4 hours before bombardment and 18 hours after	MS high osmotic medium (MS+0.2M mannitol, 0.2M sorbitol) Standard 1100 psi microcarrier discs used
Reculture	7 days	MS+2µg/mL^{-1}2,4D, 250 µg/mL^{-1}Clavamox	Reculture	7 days	MS+2µg/mL^{-1}2,4-D
Reculture	2 cycles 3-4 weeks each	MS+2µg/mL^{-1}2,4-D, 250µg/mL^{-1}Clavamox, 50µg/mL^{-1}hygromycin	Reculture	2 cycles 3-4 weeks each	MS+2µg/mL^{-1}2,4-D, 4µg/mL^{-1} bialaphos
Regeneration	1-2 weeks	MS+0.5µg/mL^{-1}zeatin, 250µg/mL^{-1}Clavamox	Regeneration	1-2 weeks	MS+0.5µg/mL^{-1}zeatin
Transfer of regenerated plants to Jiffy peat pellets	5-7 days		Transfer of regenerated plants to Jiffy peat pellets	5-7 days	
Transfer of regenerated (R^0) plants to soil	6-8 weeks		Transfer of regenerated (R^0) plants to soil	6-8 weeks	

GUS Assays

Comparative efficiency of two *Agrobacterium* preinoculation procedures of Ratnayaka (Ratnayaka, 1999) and Lichtenstein and Draper (1985) was tested and evaluated in terms of GUS expression. Efficiency was tested by exposing transformed calli pieces to X-gluc treatment 48 h after cocultivation (Table 2-3). All inoculated calli were collected and exposed to X-gluc treatment. The experiment was repeated two times (2X) for both immature and mature embryo-derived calli. Total number of calli containing blue spots with X-gluc was highest for the Lichtenstein and Draper preinoculation procedure for both immature and mature embryo-derived calli. Procedure of Lichtenstein and Draper (1985) was used for growing LBA4404 (pTOK233) culture in all subsequent *Agrobacterium*-mediated transformation experiments.

Overall results of GUS histochemical assay for immature and mature embryo-derived calli are summed up in Table 2-4. Transient GUS expression was evaluated in both *Agrobacterium*-mediated and biolistic transformation procedures 2 to 3 days after transfer to callus induction media. A significant difference in rate of transient GUS expression between immature and mature embryo-derived calli transformed with particle bombardment was observed, with former being superior. In case of *Agrobacterium*-mediated transformation, rate of transient GUS expression was similar, with immature embryo-derived calli demonstrating higher rates of uniform expression of GUS (data not shown). Examples of transient GUS expression in calli transformed by *Agrobacterium* and particle bombardment are shown in Figures 2-4 and 2-5 respectively.

Table 2-3. *Effect of two pre-inoculation procedures on transient expression of the transferred GUS gene in wheat tissues after cocultivation with A.tumefaciens LBA4404(pTOK233)*

Experiment[a]	**Preinoculation procedure of Ratnayaka (1999)**			**Preinoculation procedure of Lichtenstein and Draper (1985)**		
	Total	GUS+	%	Total	GUS+	%
1	25	3	12	24	9	37.6
2	16	12	46.1	23	18	78.2
3	15	6	40	21	9	42.8
4	22	8	36.3	23	11	47.8

[a] Experiments No 1,2 were performed with mature embryo-derived calli

Experiments No 3,4 were performed with immature embryo derived calli

GUS+ =GUS-positive

Table 2-4. *Transient GUS expression in calli transformed by either Agrobacterium or microprojectile bombardment*

Experiment No.[a]	**Microprojectile bombardment**			***Agrobacterium*-mediated transformation**		
	Total	GUS+	%	Total	GUS+	%
1	114	25	21.9	135	62	45.9
2	158	80	50.6	136	61	44.8

[a] experiment No 1 was performed with mature embryo-derived calli

experiment No 2 was performed with immature embryo-derived calli

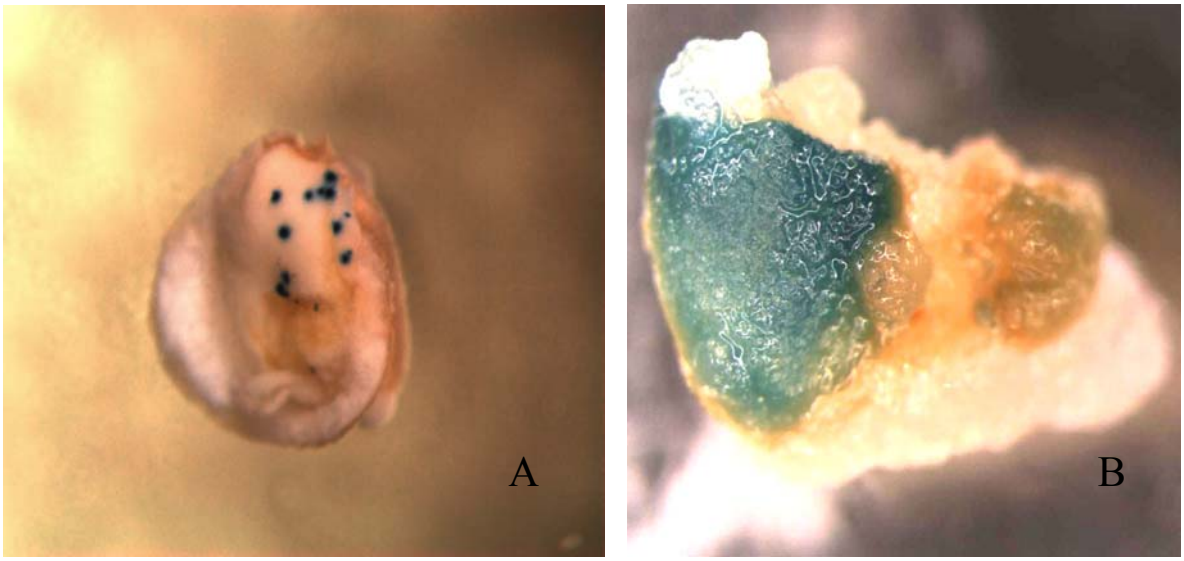

Figure 2-4. Transient GUS expression in calli inoculated with *Agrobacterium* and assayed with X-Gluc. A, An example of a pattern of GUS spots. B, An example of a uniform GUS expression. GUS spots, as well as uniform GUS expression, were mostly localized on the scutellum surface of the inoculated calli.

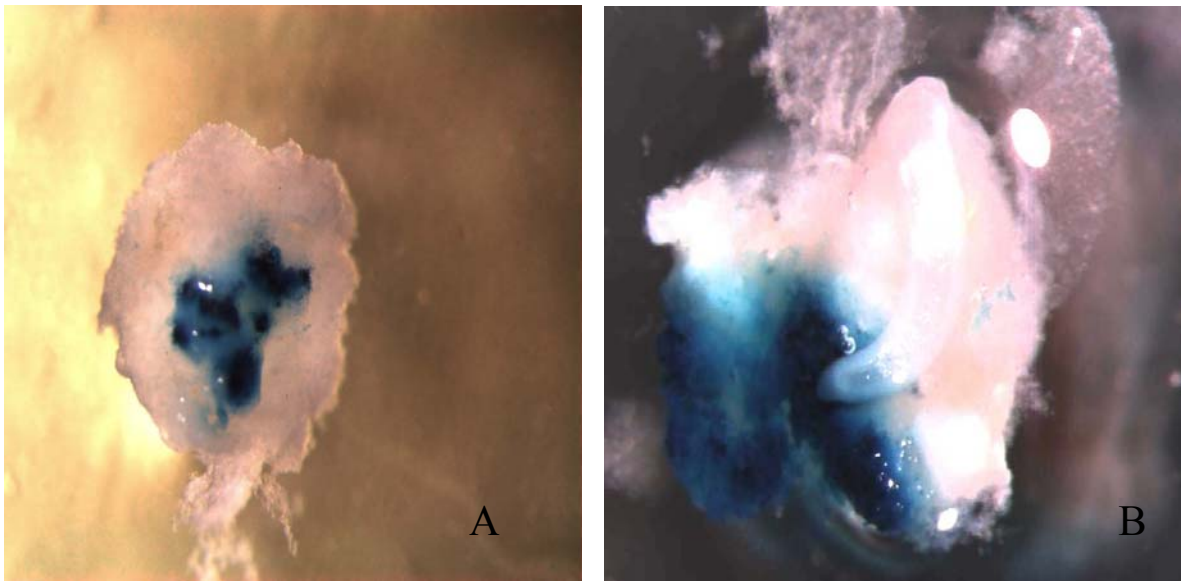

Figure 2-5. Transient GUS expression in calli transformed via particle bombardment and assayed with X-Gluc. A, An example of a pattern of GUS spots. B, An example of a uniform GUS expression.

PCR Screening

All regenerated plants were screened for the presence of GUS (*UidA), Npt, Hpt* or *Bar* genes using PCR amplification of fragments in the transferred genes. Seventeen plants were regenerated from *Agrobacterium* inoculation. Seven of the 17 plants were PCR positive for the one or more of the transformed genes. Seven plants were regenerated from the gene gun bombardment procedure. Four of the 7 plants were PCR positive for the transferred *UidA* gene. Examples of the PCR amplification reactions are shown in Figures 2-6, 2-7 (*UidA*), Figures 2-8, 2-9 (*NptII)*, Figures 2-10, 2-11 (*Hpt*), Figure 2-12 (*UidA* for gene gun bombarded plants). Results of PCR amplifications and PCR-based Southern blots for *Agrobacterium* and particle bombardment procedures are shown in Tables 2-5 and 2-6 respectively.

Plant Identification System

For *Agrobacterium* procedure, inoculated plants derived from mature embryos were labeled "S" (seeds), and plants derived from immature embryos were labeled "Em" (embryos). In PCR reactions, an additional number appears in parentheses to identify individual shoots from multiple shoots derived from the callus (e.g. S6 (2) is a DNA sample extracted from the second shoot of the mature embryo-derived plant No. 6). For plants subjected to biolistic bombardment, those derived from mature embryos were labeled Gm (gun mature), and plants derived from immature embryos were labeled Gi (gun immature).

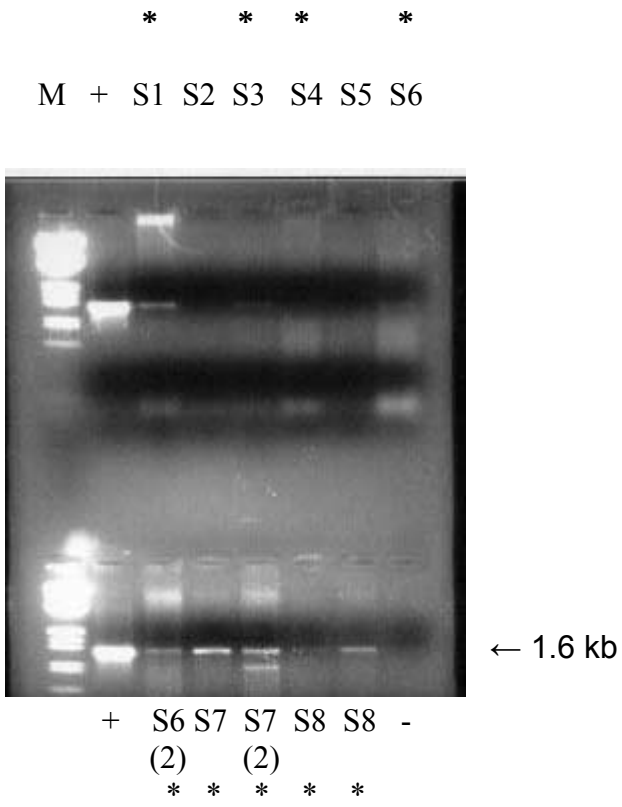

Figure 2-6. An example of PCR amplified bands of *UidA* gene in wheat plants (R^0) regenerated following *Agrobacterium* inoculation. A positive (+) control (transforming pTOK233 plasmid), a negative (-) control (non-transgenic wild type plant DNA of cv Saratovskaya-29), molecular weight markers (M) (Promega Lambda DNA markers, 21.2 kb ladder), and the expected *UidA* PCR product (1.6 kb) are indicated. DNA samples used for the PCR reaction were taken from plants regenerated from mature embryos (S1-8). * positive plants exhibiting bands of expected size.

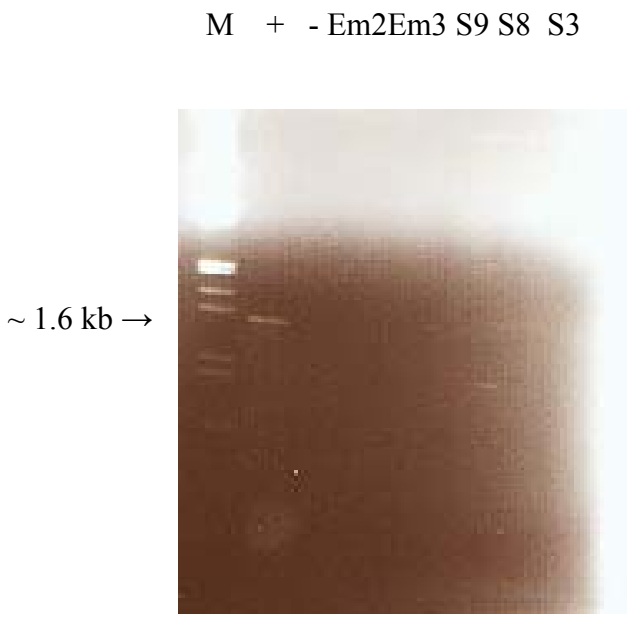

Figure 2-7. An example of PCR amplified bands of *UidA* gene in wheat plants (R^0) regenerated following *Agrobacterium* inoculation with subsequent probing for the *UidA* gene. A positive (+) control (transforming pTOK233 plasmid), a negative (-) control (non-transgenic wild type plant DNA of cv Saratovskaya-29), molecular weight markers (M) (Promega Lambda DNA markers, 21.2 kb ladder), and the expected *UidA* PCR product (~1.6 kb) are indicated.

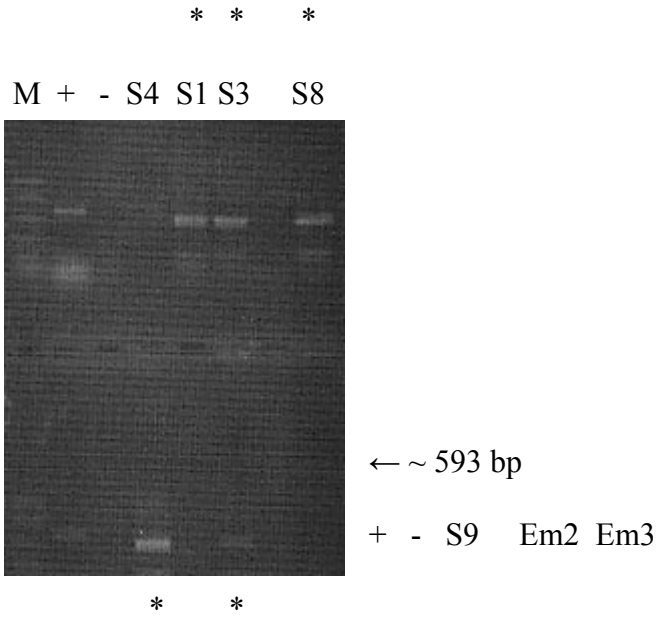

Figure 2-8. An example of PCR amplified bands of *NptII* gene in wheat plants (R^0) regenerated following *Agrobacterium* inoculation. A positive (+) control (pART27), a negative (-) control (non-transgenic plant DNA of cv Saratovskaya-29), molecular weight markers (M) (Promega PCR markers 1-kb ladder), and the expected PCR *NptII* product (~593 bp) are indicated. DNA samples used for PCR reaction were taken from plants regenerated from mature embryos (S1, S3, S4, S8 and S9) and immature embryos (Em2, Em3). * positive plants exhibiting bands of expected size.

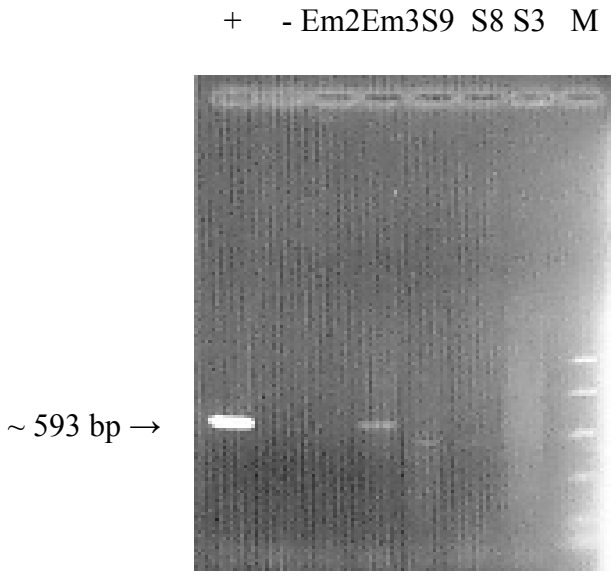

Figure 2-9. An example of PCR amplified bands of *NptII* gene in wheat plants (R^0) regenerated following *Agrobacterium* inoculation with subsequent probing for the *NptII* gene. A positive control (+) (transforming pTOK233 plasmid), a negative control (-) (non-transgenic DNA of cv Saratovskaya-29), molecular weight markers (Promega PCR markers, 1 kb ladder), and the expected PCR *NptII* product (~593 bp) are indicated.

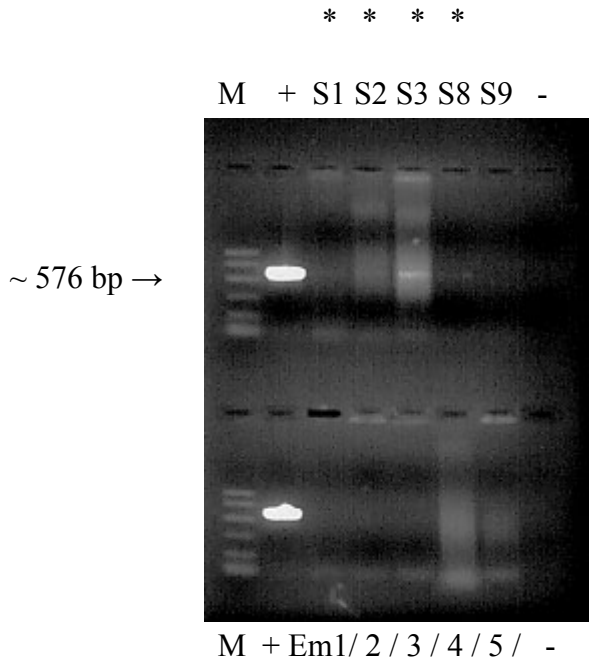

Figure 2-10. An example of PCR amplified bands of *Hpt* gene in wheat plants (R^0) regenerated following *Agrobacterium* inoculation. A positive (+) control (transforming pTOK233 plasmid), a negative control (non-transgenic wild type DNA of cv Saratovskaya-29), molecular weight markers (M) (Promega PCR markers, 1-kb ladder) and the expected PCR *Hpt* product (~ 576 bp) are indicated. DNA samples used for PCR reaction were taken from plants regenerated from mature embryos (S1, S2, S3, S8 and S9) and immature embryos (Em1, Em2, Em3, Em4 and Em5). * positive plants exhibiting bands of expected size.

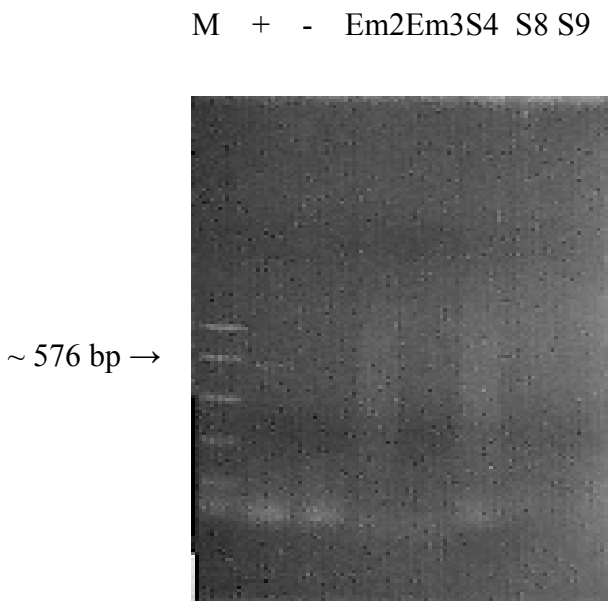

Figure 2-11. An example of PCR amplified bands of *Hpt* gene in wheat plants (R^0) regenerated following *Agrobacterium* inoculation with subsequent probing for the *Hpt* gene. A positive (+) control (transforming pTOK233 plasmid), a negative (-) control (non-transgenic DNA of cv Saratovskaya-29), molecular weight markers (M) (Promega PCR markers, 1 kb ladder), and the expected PCR *Hpt* product (~ 576 bp) are indicated.

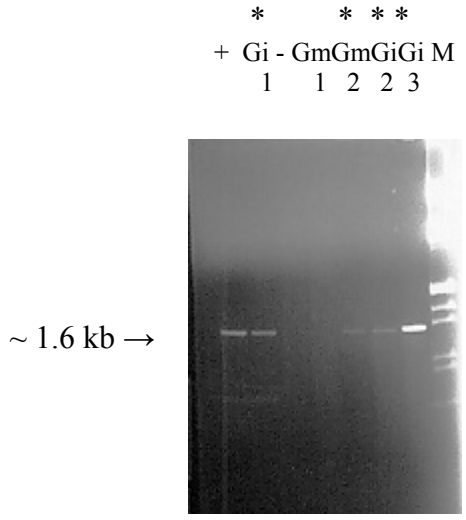

Figure 2-12. An example of PCR amplified bands of *UidA* gene in regenerated (R^0) wheat plants from callus submitted to microprojectile bombardment. A positive (+) control (transforming pAHC25 plasmid), a negative (-) control (non-transformed wild type DNA of the cv Saratovskaya-29), molecular weight markers (M) (Promega Lambda DNA markers, 21.1 kb ladder) and the expected PCR *UidA* product (~ 1.6 kb) are indicated. DNA samples for PCR reaction were taken from plants regenerated from immature embryos (Gi 1-3) and mature embryos (Gm1,Gm2). * positive plants exhibiting bands of expected size.

Table 2-5. *PCR amplification and PCR-based Southern blot of primary regenerated (R^o) plants derived from callus inoculated with Agrobacterium*

Plant Number	*Hpt* gene PCR	*Hpt* gene Hybridization	*NptII* gene PCR	*NptII* gene Hybridization	*UidA* gene PCR	*UidA* gene Hybridization
S1	+	+	+		+	
S2	-		-		-	
S3	-		+		+	
S4	-		-	-	-	-
S5	-		-		-	
S6	-		-		+	
S7	-		-		+	
S8	+	+	+	+	+	+
S9	-	+	+	+	+	+
S10	-		-		-	
S11	-		-		-	
Em1	-		-		-	
Em2	-	-	-	-	+	+
Em3	-	-	+	+	-	-
Em4	-		-		-	
Em5	-		-		-	

+ = PCR and/or Southern blot positive plants

- = plants that did not produce bands in PCR amplification reaction and/or PCR-based Southern blot

Table 2-6. *PCR amplification and PCR-based Southern blot of primary regenerated (R^0) plants transformed via particle bombardment*

Plant number	***UidA* PCR**	***UidA* Hybridization**	***Bar* PCR**	***Bar* Hybridization**
Gi1	+	+	-	-
Gi2	+	+	-	-
Gi3	+	+	-	-
Gi4	-		-	-
Gm1	-	-	-	-
Gm2	+	+	-	-
Gm3	-		-	-

+ = PCR positive plants

- = plants that did not produce bands in PCR amplifications and/or PCR-based Southern blot

Molecular Analysis of Regenerated Plants

Five plants regenerated following *Agrobacterium* inoculation and five plants regenerated following microprojectile bombardment were subjected to the PCR amplification of the transferred genes and Southern blotting of PCR amplified fragments. PCR amplified *UidA* gene fragment from plasmid pTOK233 was used as a probe for the *UidA* gene in PCR amplified DNA of *Agrobacterium*-transformed plants, and the PCR amplified *UidA* fragment from plasmid pAHC25 was used as a probe for the *UidA* gene in PCR amplified DNA of microprojectile bombarded plants. DNA from plasmid pG35BarB (Rathore et al., 1993), PCR amplified with *Bar* gene primers, was used as a probe for the *Bar* gene in PCR amplified DNA of microprojectile bombarded plants. DNA from plasmid pCAM1300, PCR amplified with *Hpt* primers, and DNA from plasmid pART27 (Gleave, 1992), PCR amplified with *NptII* primers, was used as probes for the *Hpt* and *NptII* genes respectively in *Agrobacterium*-transformed R^0 plants.

Hpt Gene

Results of PCR-based Southern blot analysis of R0 regenerated plants for the presence of *hpt* gene are shown in Figure 2-13. As it was noted, the *hpt* probe was PCR amplified DNA from *Hpt* gene of plasmid pCAM1300. Non-transformed DNA from wheat plants of cv Saratovskaya-29 was used as a negative control, and PCR amplified DNA from plasmid pCAM1300 was used as a positive control. Plants S8 (lane 5) and S9 (lane 7), both mature embryo derived, showed fragments that hybridized to the probe.

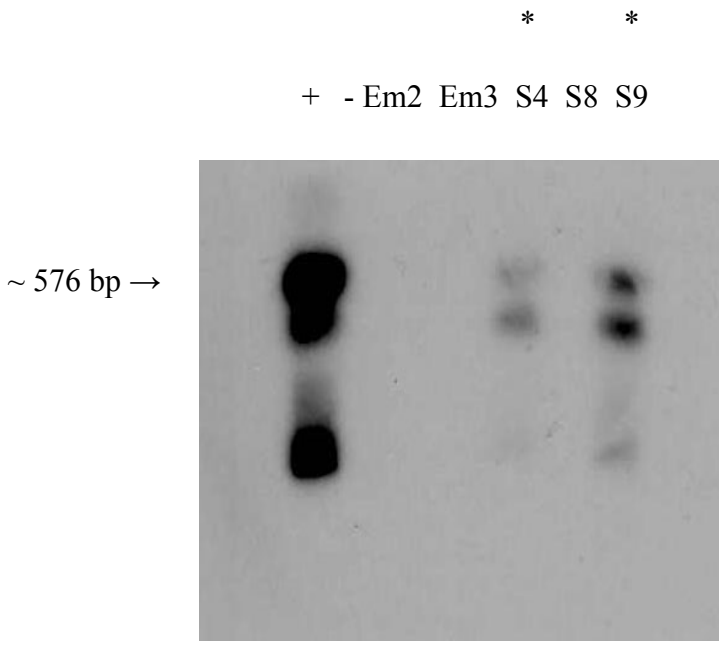

Figure 2-13. PCR-based Southern blot analysis of regenerated wheat plants (R^0) probed for the *Hpt* gene. Positive control (+) (PCR amplified DNA of plasmid pCAM 1300), negative control (-) (non-transgenic DNA of cv Saratovskaya-29), and the amplified *Hpt* fragment of the expected size (~576 bp) are indicated. Southern blot analysis was performed on *Hpt* gene PCR gel shown in Fig. 2-11. * positive plants exhibiting presence of the *Hpt* gene.

NptII Gene

Results of PCR-based Southern blot analysis of R^0 regenerated wheat plants for the presence of the *NptII* gene are shown in figure 2-14. The probe for the *NptII* gene was prepared using PCR amplified product from the plasmid pART27, which was purified using MinElute PCR Purification Kit Protocol (MinElute Handbook, QIAGEN, 2001) and then used for the preparation of the radioactive probe. Non-transformed DNA of cv Saratovskaya-29 was used as a negative control, and PCR amplified DNA of the plasmid pART27 was used as a positive control. Fragments of the DNA from plants Em2 (lane 3, immature embryo derived plant) and S9 (lane 5, mature embryo derived plant) hybridized to the probe.

UidA Gene

Figure 2-15 shows the results of the PCR-based blot analysis of *Agrobacterium* transformed plants for the presence of *UidA* gene. The probe for the *UidA* gene was prepared using PCR amplified product from the plasmid pTOK233 which was further purified using MinElute PCR Purification Kit Protocol. Non-transformed DNA of cv Saratovskaya-29 was used as a negative control, and PCR amplified DNA of the plasmid pTOK233 was used as a positive control. Fragments of the DNA from plants Em2 (lane 4, immature embryo derived plant), S9 (lane 6, mature embryo derived plant), and S8 (lane 7, mature embryo derived plant) hybridized to the probe. Plant Em2 shows faint band in comparison with other *UidA* positive plants and positive control, suggesting insertion of fewer copies of the gene during the transformation process.

UidA Gene (Microprojectile Bombardment Procedure)

Fig. 2-16 shows results of the PCR-based Southern blot of the regenerated (R^0) plants transformed via microprojectile bombardment and probed for the *UidA* gene. The probe for the *UidA* gene was prepared using PCR amplified product from the plasmid pAHC25 further purified using MinElute PCR Purification Kit Protocol. Non-transformed DNA of cv Saratovskaya-29 was used as a negative control, and PCR amplified DNA of the plasmid pAHC 25 was used as a positive control. Fragments of the DNA from the plants Gi1 (lane 2, immature embryo derived plant), Gm2 (lane 5, mature embryo derived plant), Gi2 (lane 6, immature embryo derived plant), and Gi3 (lane 7, immature embryo derived plant) hybridized to the probe.

It should be noted that no hybridization to the *Bar* probe was obtained during the PCR-based Southern blot of the regenerated (R^0) plants transformed via particle bombardment, except a single case. The probe for the *Bar* gene was prepared using PCR amplified product from pG35barB plasmid (Rathore et al., 1993), supplied courtesy of Dr. Rathore.

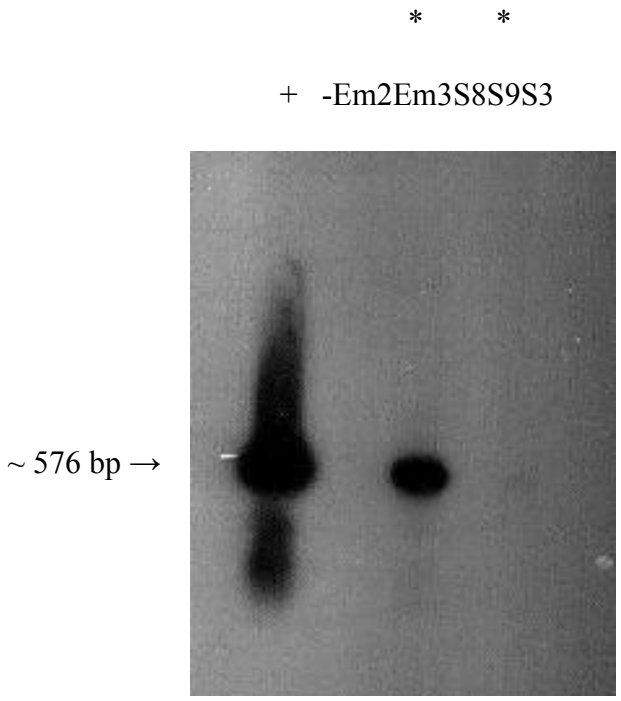

Figure 2-14. PCR-based Southern blot analysis of regenerated wheat plants (R^0) probed for the *NptII* gene. Positive control (+) (PCR amplified DNA of plasmid pART27), negative control (non-transgenic DNA of cv Saratovskaya-29), and the *NptII* amplified fragment of expected size (~ 576 bp) are indicated. Southern blot analysis was performed on *NptII* gene PCR gel shown in Fig. 2-9. * positive plants exhibiting presence of the *NptII* gene.

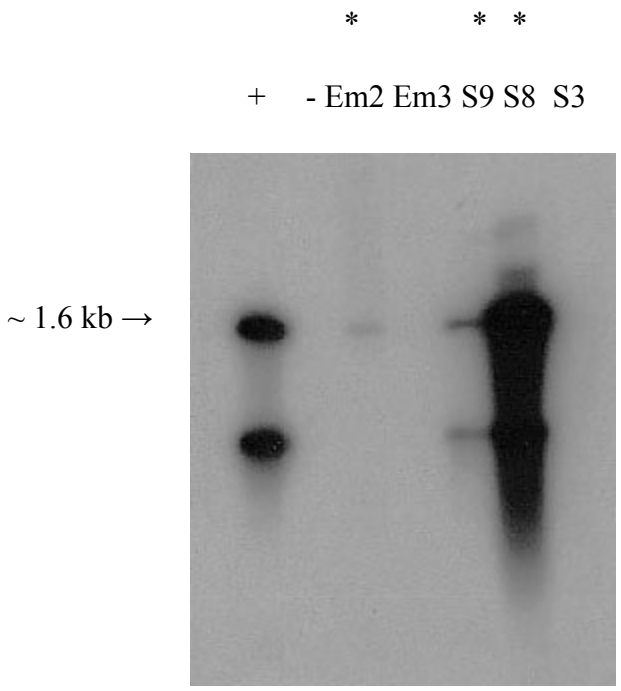

Figure 2-15. PCR-based Southern blot analysis of regenerated wheat plants (R^0) probed for the *UidA* gene. Positive control (+) (PCR amplified DNA of plasmid pTOK233), negative control (non-transgenic DNA of cv Saratovskaya-29), and *UidA* amplified fragment of expected size (~1.6 kb) are indicated. Southern blot analysis was performed on *UidA* gene PCR gel shown in Fig. 2-7. * positive plants exhibiting presence of the *UidA* gene.

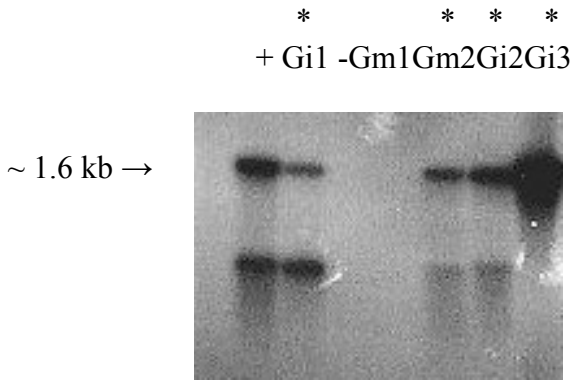

Figure 2-16. PCR-based Southern blot analysis of regenerated wheat plants (R^0) transformed via particle bombardment and probed for *UidA* gene. Positive control (+) (PCR amplified DNA of plasmid pAHC25), negative control (-) (non-transgenic DNA of cv Saratovskaya-29), and the *UidA* amplified fragment of expected size (~ 1.6 kb) are indicated. Southern blot analysis was performed on *UidA* gene PCR gel shown in Fig. 2-12. * positive plants exhibiting presence of the *UidA* gene.

R^1 Generation

To verify inheritance of the transferred genes, DNA from the developing shoots and/or embryos of the R^1 progeny of the 9 R^0 plants was extracted, using urea-phenol extraction method (Shure et al., 1983) and PCR amplified for the presence of the transferred genes. Progeny of 5 mature embryo-derived R^0 plants transformed with *Agrobacterium* (S1, S2, S3, S6 and S9) 2 immature-embryo-derived R^0 plants transformed with *Agrobacterium* (Em2, Em3), and 2 R^0 plants transformed with microprojectile bombardment (Gi2, Gm2) was evaluated. Results of the PCR amplification of the *UidA*, *NptII* and *Hpt* genes in the progeny of the plant S9 are shown in Figures 2-17, 2-18, and 2-19 respectively. With 4 progeny tested, 3 were PCR positive for the amplified *UidA* fragment of expected size (~ 1.6 kb), 4 were PCR positive for the amplified *NptII* fragment, and 3 were PCR positive for the amplified *Hpt* fragment. Results of the PCR-based Southern blot analysis of the progeny of plant S9 for the presence of the *UidA, NptII* and *Hpt* genes are shown in Figures 2-20, 2-21, and 2-22 respectively. PCR-based Southern blot analysis of DNA of the 4 progeny of the S9 plant showed progeny plants S9-1, S9-3 and S9-4 hybridizing to *UidA* probe, plants S9-1, S9-2, S9-3, and S9-4 hybridizing to *NptII* probe, and plants S9-2, S9-3, and S9-4 hybridizing to *Hpt* probe. Results of these PCR-based Southern blots might suggest possible inheritance of the transferred genes in R^1 progeny of the S9 plant. Results of the PCR amplification of the *UidA, NptII* and *Hpt* genes in the progeny of the plant Em2 are shown in Figures 2-23, 2-24, and 2-25 respectively. With 10 progeny tested, 4 were

PCR positive for the amplified *UidA* gene fragment, 9 were PCR positive for the amplified *NptII* gene fragment, and 8 were PCR positive for the amplified *Hpt* fragment. Results of PCR amplification of the *UidA* and *Bar* genes in the progeny of the plant Gi2 are shown in Figures 2-26 and 2-27 respectively. With 8 progeny tested, 1 was PCR positive for the amplified *Bar* gene fragment, and 7 were PCR positive for the amplified *UidA* fragment.

Overall results of the PCR amplification of the transferred genes in the progeny of the tested R^0 plants transformed with *Agrobacterium* inoculation or microprojectile bombardment are summed up in Tables 2-7 and 2-8 respectively. Although use of PCR-based Southern blot procedure in the study does not allow suggesting number of gene copies inserted as a result of transformation procedure applied, progeny of all 9 R^0 plants tested were PCR positive for one or more genes transferred, thus suggesting a possibility that both genetic constructs were transferred and inherited. Total number of PCR positive R^0 plants with PCR positive R^1 progeny is shown in Table 2-9.

Young shoots and roots of R^1 progeny of R^0 PCR positive plants were subjected to X-gluc assay. No GUS expression was observed with material of all 7 plants tested, whereas PCR amplification of the *UidA* gene was observed in R^1 progeny of all these plants. Absence of GUS expression in the R^1 progeny might be explained by possible silencing of the *UidA* gene. Absence of the GUS expression in R^1 progeny of primary transformed plants was observed before and several hypotheses explaining this event were suggested (Demeke et al., 1999).

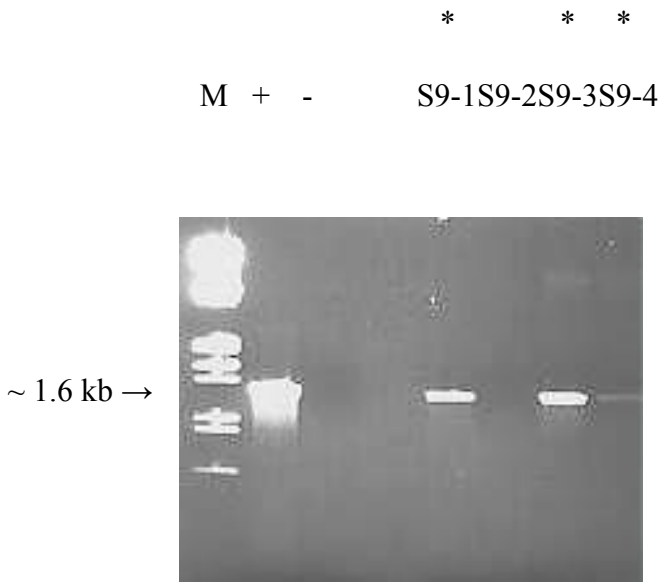

Figure 2-17. PCR amplified bands of *UidA* gene in the R^1 generation progeny of the wheat plant S9. Positive (+) control (transforming pTOK233 plasmid), negative (-) control (no DNA template), molecular weight markers (M) (Promega Lambda DNA markers, 21.1 kb-ladder) and the *UidA* amplified fragment of expected size (~1.6 kb) are indicated. * positive plants exhibiting bands of expected size.

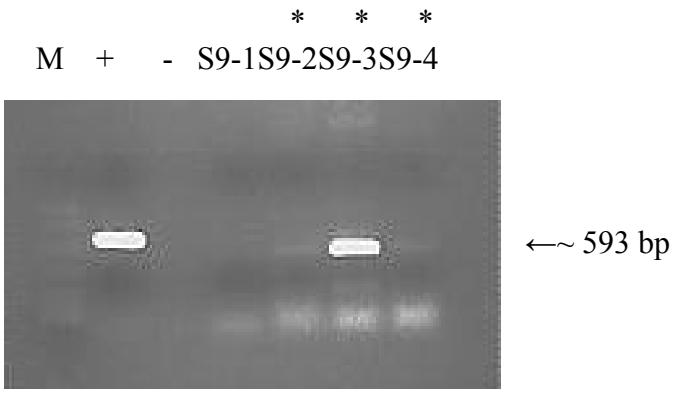

Figure 2-18. PCR amplified bands of *NptII* gene in the R^1 generation progeny of the wheat plant S9. Positive control (+) (transforming pTOK233 plasmid), negative control (-) (no DNA template), molecular weight markers (M)(Promega PCR markers, 1kb-ladder), and the amplified *NptII* band of expected size are indicated. * positive plants exhibiting bands of expected size.

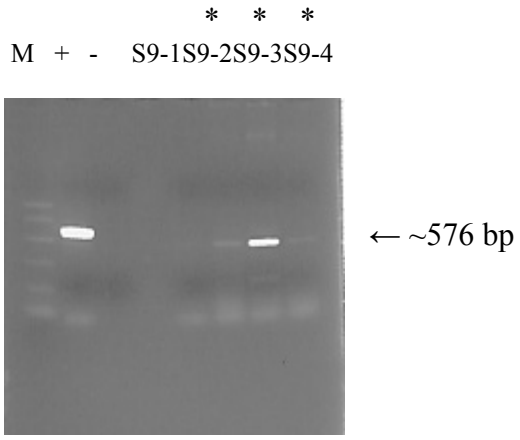

Figure 2-19. PCR amplified bands of *Hpt* gene in the R^1 generation progeny of the wheat plant S9. Positive control (+) (transforming pTOK233 plasmid), negative control (-) (no DNA template), molecular weight markers (Promega PCR markers, 1-kb ladder), and the amplified *Hpt* fragment of the expected size (~ 576 bp) are indicated. * positive plants exhibiting bands of expected size.

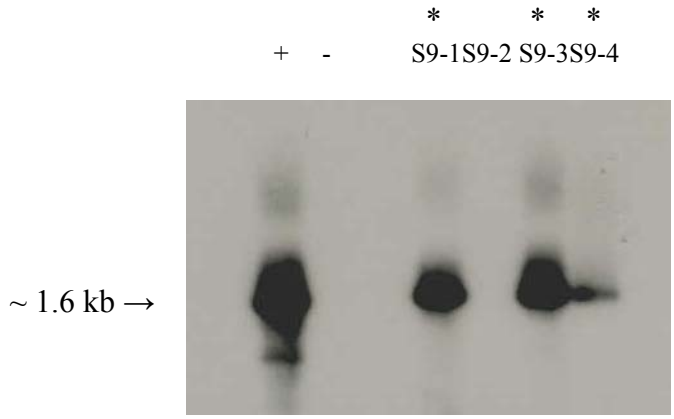

Figure 2-20. PCR-based Southern blot analysis of R^1 generation progeny of the wheat plant S9 probed for the *UidA* gene. Positive control (+) (transforming plasmid pTOK233), negative control (-) (no DNA template), and *UidA* amplified fragment of expected size (~ 1.6 kb) are indicated. Southern blot analysis was performed on *UidA* gene PCR gel shown in Fig. 2-17. * positive plants exhibiting presence of the *UidA* gene.

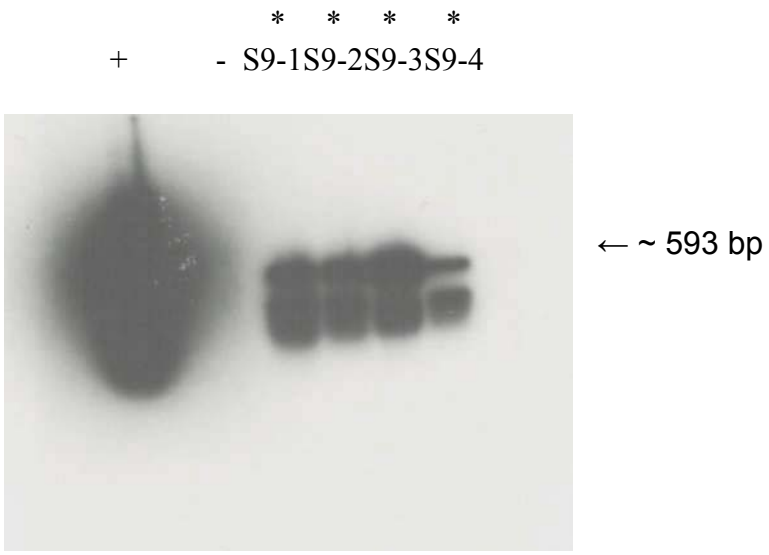

Figure 2-21. PCR-based Southern blot analysis of R^1 generation progeny of the wheat plant S9 probed for the *NptII* gene. Positive control (+) (transforming pTOK233 plasmid), negative control (-) (no DNA template), and *NptII* amplified fragment of expected size (~593 bp) are indicated. Southern blot analysis was performed on *NptII* gene PCR gel shown in Fig. 2-18. * positive plants exhibiting presence of the *NptII* gene.

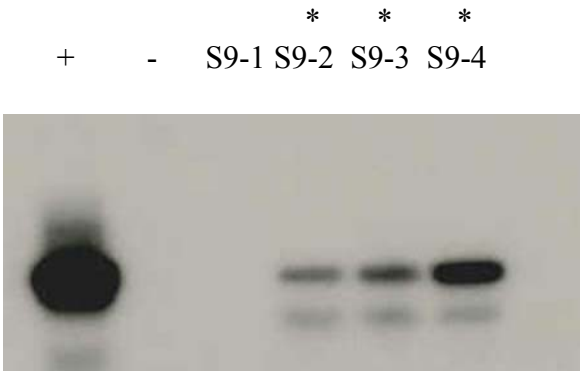

Figure 2-22. PCR-based Southern blot analysis of R^1 generation progeny of the wheat plant S9 probed for the *Hpt* gene. Positive control (+) (transforming pTOK233 plasmid), negative control (-) (no DNA template), and amplified *Hpt* fragment of expected size (~ 576 bp) are indicated. Southern blot analysis was performed on *Hpt* gene PCR gel shown in Fig. 2-19. * positive plants exhibiting presence of the *Hpt* gene.

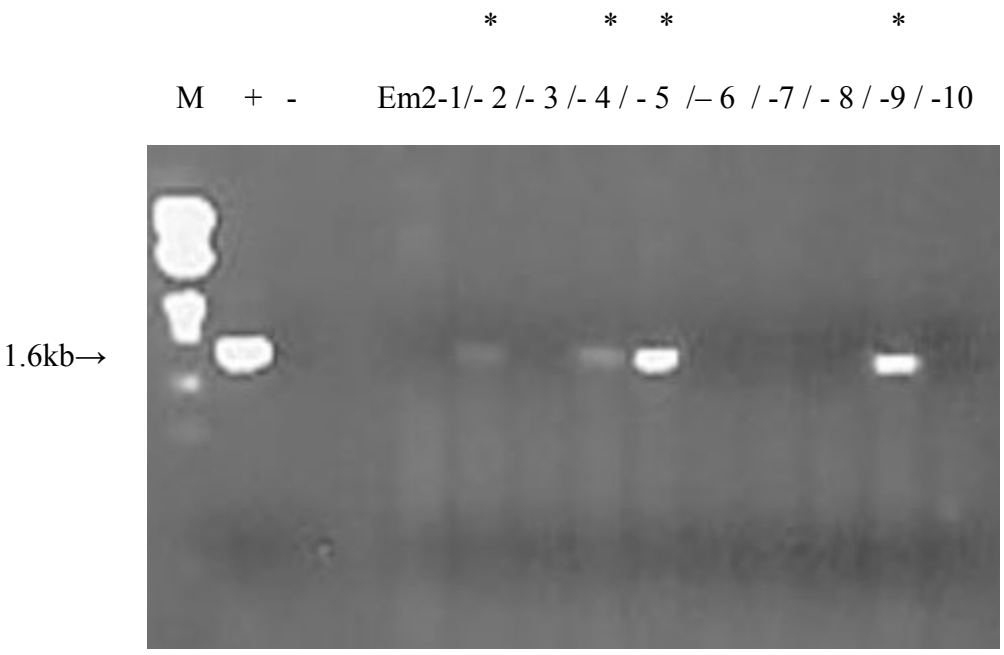

Figure 2-23. PCR amplified bands of *UidA* gene in the R^1 generation progeny of the wheat plant Em2. Positive control (+) (transforming plasmid pTOK233), negative control (-) (no DNA template), molecular weight markers (M) (Promega Lambda DNA Markers, 21.1-kb ladder), and the *UidA* amplified band of expected size (~1.6 kb) are indicated. * positive plants exhibiting bands of expected size.

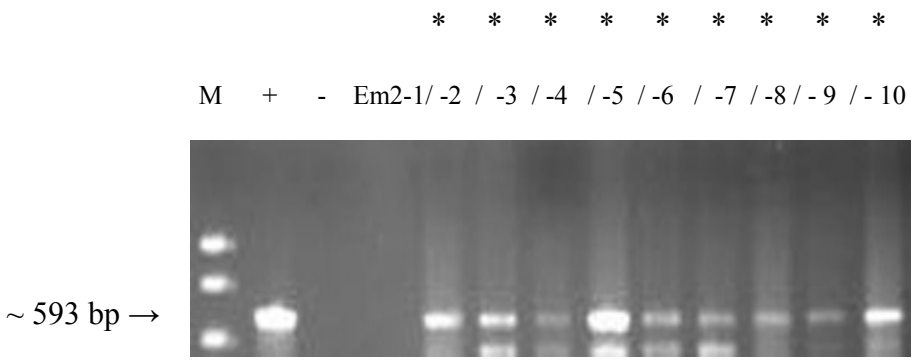

Figure 2-24. PCR amplified bands of *NptII* gene in the R^1 generation progeny of the wheat plant Em2. Positive control (+) (transforming pTOK233 plasmid), negative control (-) (no DNA template), molecular weight markers (M) (Promega PCR markers, 1-kb ladder), and *NptII* amplified fragment of the expected size (~ 593 bp) are indicated. * positive plants exhibiting bands of expected size.

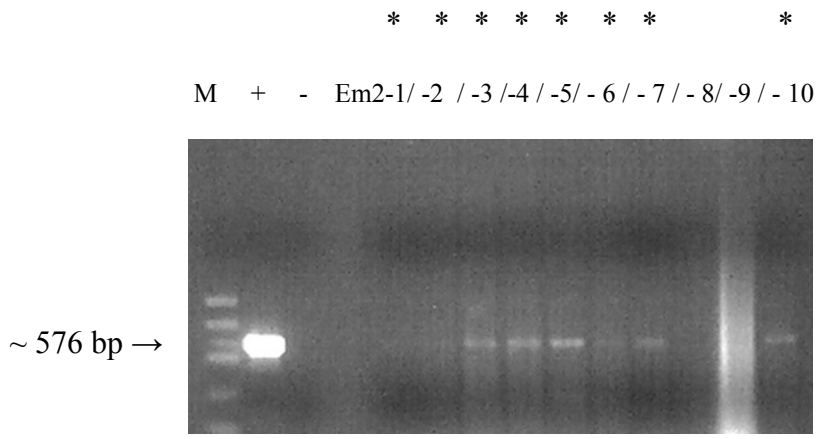

Figure 2-25. PCR amplified bands of *Hpt* gene in R^1 generation progeny of the wheat plant Em2. Positive control (+) (transforming plasmid pTOK233), negative control (-) (no DNA template), molecular weight markers (M) (Promega PCR Markers, 1-kb ladder), and the *Hpt* amplified band of expected size (~ 576 bp) are indicated. * positive plants exhibiting bands of expected size.

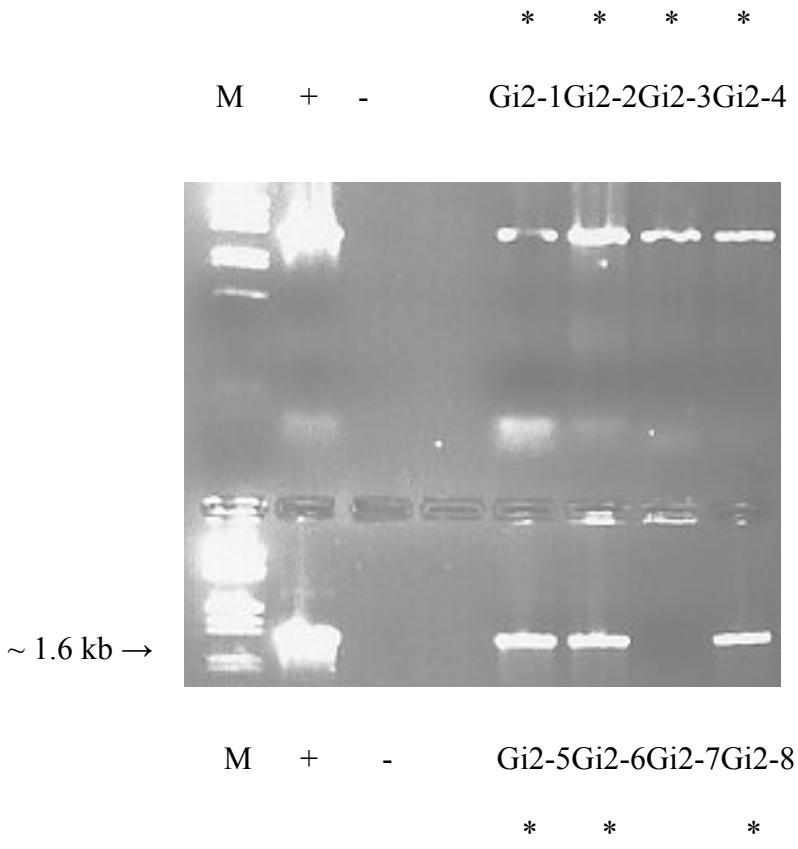

Figure 2-26. PCR amplified bands of *UidA* gene in R^1 generation progeny of wheat plant Gi2. Positive control (+) (transforming plasmid pAHC25), negative control (-) (no DNA template), molecular weight markers (M) (Promega Lambda DNA Markers, 21.1 kb- ladder) and *UidA* amplified band of expected size (~ 1.6 kb) are indicated. * positive plants exhibiting bands of expected size.

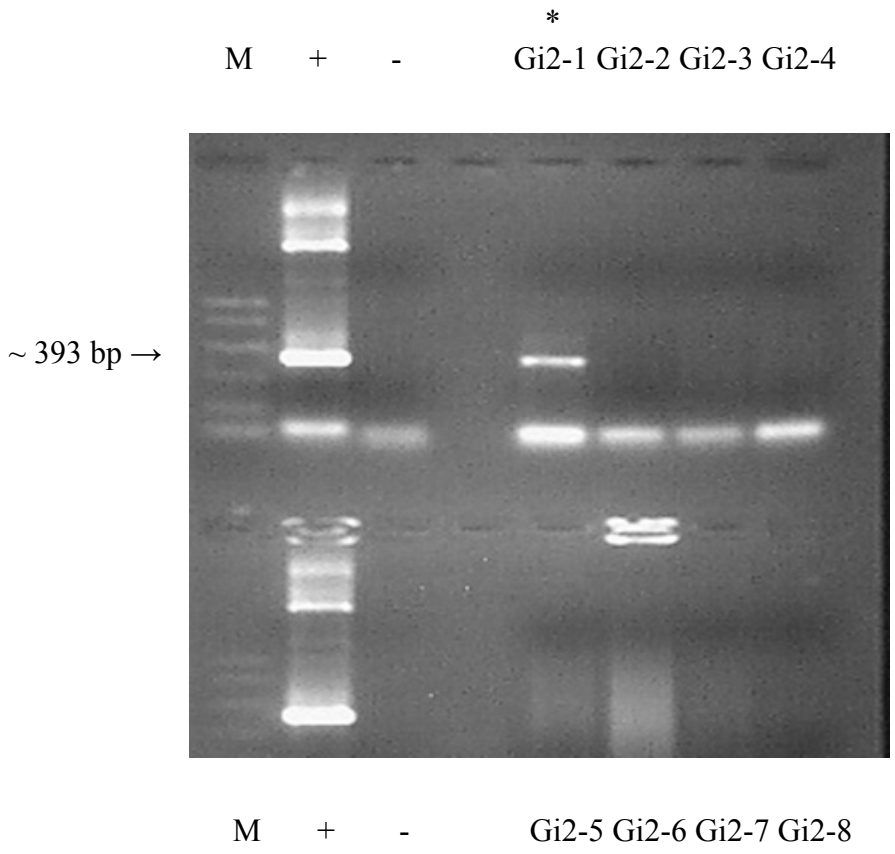

Figure 2-27. PCR amplified bands of *Bar* gene in R^1 generation progeny of the wheat plant Gi2. Positive control (+) (PG35BarB plasmid), negative control (-) (no DNA template), molecular weight markers (M) (Promega PCR markers, 1-kb ladder), and *Bar* amplified band of the expected size are indicated. * positive plants exhibiting bands of expected size.

Table 2-7. *PCR amplification of R^1 progeny of plants inoculated with Agrobacterium*

No of the plant progeny	*UidA* gene PCR	*NptII* gene PCR	*Hpt* gene PCR
S1-1	+	+	+
S1-2	-	-	-
S1-3	-	-	-
S1-4	+	+	-
S2-1	-	+	-
S2-2	-	-	-
S2-3	-	-	-
S2-4	-	-	+
S2-5	+	+	+
S3-1	+	+	-
S3-2	-	+	-
S3-3	+	+	+
S4-3	+	+	+
S6-1	+	+	-
S6-2	-	-	-
S6-3	-	-	+
S6-4	-	-	-

Table 2-7. *Continued.*

No of the plant progeny	*UidA* gene PCR	*NptII* gene PCR	*Hpt* gene PCR
S9-1	+	-	-
S9-2	-	+	+
S9-3	+	+	+
S9-4	+	+	+
Em2-1	-	-	+
Em2-2	+	+	+
Em2-3	-	+	+
Em2-4	+	+	+
Em2-5	+	+	+
Em2-6	-	+	+
Em2-7	-	+	+
Em2-8	-	+	-
Em2-9	+	+	-
Em2-10	-	+	+
Em3-1	-	-	-
Em3-2	-	-	-
Em3-3	-	-	-
Em3-4	-	-	-
Em3-5	-	+	-

Table 2-7. *Continued.*

No of plant progeny	*UidA* gene PCR	*NptII* gene PCR	*Hpt* gene PCR
Em3-6	-	+	-
Em3-7	-	-	-
Em3-8	+	+	-

Table 2-8. *PCR amplification of R^1 progeny of plants transformed with particle bombardment*

No of the plant progeny	*UidA* gene PCR	*Bar* gene PCR
Gi2-1	+	+
Gi2-2	+	-
Gi2-3	+	-
Gi2-4	+	-
Gi2-5	+	-
Gi2-6	+	-
Gi2-7	-	-

Table 2-8. *Continued.*

No of the plant progeny	*UidA* gene PCR	*Bar* gene PCR
Gi2-8	+	-
Gm2-1	-	-
Gm2-2	-	-
Gm2-3	-	-
Gm2-4	+	-
Gm2-5	-	-
Gm2-6	+	-
Gm2-7	-	-
Gm2-8	-	-
Gm2-9	-	-
Gm2-10	-	-

Table 2-9. *Total number of PCR positive R^0 plants with PCR positive R^1 progeny*

Procedure	Number of plants[a]	R^0 PCR+[b]	R^0 PCR Blot[b]	Number of R^0 with R^1 PCR+[b,c]	Rate: PCR+ R^0 with R^1 PCR+[a]
Agrobacterium					
Mature embryos	11	6	5	4	4/452=0.88%
Immature embryos	6	1	1	1	1/622=0.16%
Particle Bombardment					
Mature embryos	3	1	1	1	1/460=0.22%
Immature embryos	4	3	3	3	3/526=0.57%

[a] values from Table 2-2
[b] values from Table 2-5 and 2-6
[c] values from Table 2-7 and 2-8

DISCUSSION

The principal goal of this study was to determine the applicability of two most widely used transformation techniques to transfer foreign genes into the genome of an elite Russian spring wheat cultivar Saratovskaya-29. As it was noted, this cultivar was one of the most common parents of modern Kazakhstani spring wheat cultivars (Martynov and Dobrotvorskaya, 1996). Results of the PCR amplification analysis of the R^0 original transformed plants and R^1 progeny along with PCR-based Southern blot analysis suggest that genomic integration and inheritance of the transferred genes might have taken place with both *Agrobacterium*-mediated and particle bombardment transformation procedures. Two sources of tissue explants were used in the study-immature and mature embryo-derived calli. Putative transgenic R^0 plants with probable inheritance of one or more transferred genes by R^1 generation were obtained from both types of explants used in the study. Successful transformation of wheat using *A. tumefaciens* was first reported by Cheng et al. (1997). Model cultivar "Bobwhite" was used in this study, as well as in several other works (Weeks et al., 1993; Becker et al., 1994; Hu et al., 2003). Problem of adjustment of well-established transformation techniques for routine use with elite cultivars is of importance, in author's opinion. Therefore, in the study described, attempt was made to develop routine transformation and regeneration protocol for elite cultivar allowing for transferred genes to be inherited stably and in predictable manner. Our study produced first transgenic progeny for the cultivar Saratovskaya-29. Comparison of two pre-inoculation procedures performed in the study allowed to discover that procedure by Lichtenstein and Draper (1985) gives

higher rates of *UidA* gene transient expression, which might have been a result of increased virulence of the *A.tumefaciens* strain LBA4404, used in the study. Regeneration protocol that allowed producing fertile plants from transformed tissue explants was developed, with plant recovery reaching 1.58% for *Agrobacterium*-mediated procedure, and 0.7% for particle bombardment procedure. All obtained plants were fertile and produced viable seeds. It should be noted that length of culture time was increased in comparison with the field growth, which might be explained by the use of callus-based method, having additional dedifferentiation developmental step, and post-transformational stress.

Molecular analysis of the R^0 and R^1 plants revealed no PCR amplification or hybridization of the *Bar* gene, except a single case in R^1 progeny of plant Gi2, whereas PCR results for the presence of *UidA* gene were positive for both R^0 and R^1 generation plants. But R^0 plants, obtained in the particle bombardment procedure, were developing on bialaphos-containing media, suggesting that *Bar* gene was transferred and was being expressed. Most likely explanation for this observation might be the PCR artifact, caused by non-optimal conditions of the PCR reaction that were used for the amplification of the *Bar* gene, preventing efficient amplification of the *Bar* sequence. Also, additional bands were observed in several cases along with the expected band of the transferred gene. Suboptimal PCR conditions, such as improper annealing temperature, unsuitable primers or excessive number of amplification cycles used might have caused appearance of these additional bands as well. PCR reactions were performed for the detection of the

presence of transferred genes. Genomic Southern blot is required for answering the question about the origin of these additional bands.

Kohil et al. (1999) discovered 19 bp palindromic sequence inside CaMV35S promoter that might act as a recombination hotspot. In the study of Ratnayaka (1999), loss of *UidA* gene was observed with plasmid pTOK233 used for *Agrobacterium*-mediated transformation. Alignment of the amplified fragment of one of the R^1 plants with sequence of 35S promoter allowed to discover loss of the part of the 35S sequence in the plant genomic DNA. Recombination that occurred between 35S promoter sites was suggested as cause of the deletion of the part of 35S promoter sequence and fragment containing *UidA* gene. Author suggests that the design of the vector (pTOK233) with two duplicated 35S sites might have caused the loss of *UidA* gene. The same reason might have caused loss of *UidA* gene in several progeny plants of Em2 plant (particularly, Em-3, Em-6, Em-7, Em-8 and Em-10, where *NptII* and *Hpt* genes were amplified by PCR procedure). However, PCR amplification of the DNA of R^1 progeny of several regenerated plants (particularly, S1, S2 and S6 progeny was used) with CaMV35S forward and reverse primers revealed amplification of ~1.8 kb expected fragment approximately including 35S promoter sites. This fragment was observed both in pTOK233 plasmid and in R^1 progeny plant DNA, thus suggesting that the 35S promoter passed into R^1 generation of *Agrobacterium*-transformed plants intact (data not shown). Thus, deletion of a part of 35S sequence did not occur in the mature embryo-derived plants tested.

Agrobacterium-mediated transformation had several advantages in the study described. Regeneration of *Agrobacterium*-inoculated plants required less time than for those transformed via particle bombardment, and grain yield was also higher in most cases. Results of PCR amplification of DNA of R^1 progeny of the original transgenic plants, obtained via *Agrobacterium*-mediated transformation, for the presence of the transferred genes suggests inheritance of these genes for all plants tested. Data obtained suggest further use of *Agrobacterium*-mediated method for the transformation of the cultivar Saratovskaya-29 as a main transformation method.

CHAPTER III

CONCLUSIONS

To our knowledge, this is the first demonstration of putative transformation of cultivar Saratovskaya-29. One of the most widely used explant sources for crop plant transformations is callus derived from somatic tissues. Our research suggests the applicability of callus and regeneration of plants via organogenesis in the establishment of a transformation method for the elite wheat cultivar Saratovskaya-29, supporting previous reports on successful transformation of other wheat varieties using callus as an explant source (Vasil et al., 1992; Altpeter et al., 1996; Cheng et al., 1997; Weir et al., 2001; Khanna and Daggard, 2003). Use of the pTOK233 plasmid for the *Agrobacterium*-mediated transformation of wheat demonstrated that this genetic construct, successfully used earlier for the *Agrobacterium*-mediated transformation of rice (Hiei et al., 1994; Ratnayaka et al., 1999), could be stably transformed into wheat genome as well. Amplification of the *UidA* gene was especially evident in both R^0 and R^1 generations of transformants, though several cases of absence of amplification of *UidA* gene were observed. Earlier cases of loss of *UidA* gene in R^1 and R^2 progeny of rice transformed with pTOK233 plasmid due to homologous recombination between two CaMV35S promoter sites (Ratnayaka, 1999) weren't observed in our study, and presence of 35S promoter fragments was observed in amplification reactions of R^1 progeny of original transformants.

Sterility is a frequently observed problem in plants regenerated from callus; however in this study, all plants recovered from both transformations procedures were fertile and produced viable progeny, although grain yield was lower in comparison with wild type plants (data not shown). Phenotypic abnormalities observed with the original transformants suggest that somaclonal variations might have taken place during the prolonged tissue culture stage of the development of transferred explants. It might be important to develop regeneration protocol allowing decrease in the longevity of tissue culture step with a view to avoid increase in somaclonal variation.

Our study has shown a slight superiority of an *Agrobacterium*-mediated transformation method in terms of overall plant recovery ratio and the number of PCR-positive plants obtained. Genomic blot analyses of this progeny generation are needed to determine if the transferred genes, detected in the DNA by PCR, have actually been incorporated into chromosomal DNA.

This approach to transformation might be suggested for further use with the cultivar studied, to transfer beneficial genes to this cultivar. Specifically, the Saratovskaya-29 cultivar suffers from susceptibility to fungal diseases, such as leaf rust (*Puccinia recondita f.* ssp. *triticii*) and mildew. Introduction of fungal resistance gene(s) might aid this cultivar in terms of grain yield and resistance. In a recent study, cDNAs encoding antifungal protein Ag-AFP (barley class II chitinase) from *Aspergillus giganteus* were shown to be introduced and expressed in wheat, causing significant reduction in formation of leaf rust and powdery mildew (Oldach et al., 2001).

Both immature and mature embryo-derived calli might be used for transformation, since in our study putative transgenic plants were regenerated from both types of explant source. Also, use of shoot apices as an explant source might be suggested, since in this case time span for the tissue culture step would be significantly decreased, and the inoculation process might be more effective. Unlimited dedifferentiation ability of meristematic cells makes them an appropriate target for the plant transformation; meristematic cells were already used successfully for the transformation of such major crop plants as maize (Gould et al., 1991) and rice (Chan et al., 1993). Transformation of the cultivar Saratovskaya-29 using calli and other explants such as the shoot apex (Smith et al., 1992) or floral dip (Dong et al., 2001) might be an important additional step in discovering most efficient method of transformation for the described cultivar.

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VITA

Name: Arman A. Kopbayev

Education: B.S., 1997, Biology and Geography

Zhezkazgan University, Zhezkazgan, Kazakhstan

Winner of Governmental "Bolashak" scholarship for M.S. studies

M.S., 2004, Molecular and Environmental Plant Sciences

Texas A&M University, College Station, TX 77843, USA

Permanent address: 5 Esenberlin Str., Apt.9, 477000 Zhezkazgan, Kazakhstan.